

**Genetic Analysis of**  
*Phytophthora infestans*

**Theo van der Lee**

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**Genetic analysis of *Phytophthora infestans***

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# CHAPTER 1

## GENERAL INTRODUCTION



## POTATO LATE BLIGHT AND ITS IMPACT ON THE DEVELOPMENT OF PLANT PATHOLOGY

SINCE its introduction in Europe in the sixteenth century by Spanish globe-trotters, the popularity of potato, *Solanum tuberosum*, increased steadily and in the nineteenth century potato had become the staple food for Western Europe. The dependence on potato was dreadfully visualized during the outbreak of diseases in 1845. Dramatic losses of yield caused famine in large parts of Western Europe, particularly in Ireland, and as a consequence over a million people died (BOURKE 1993). At the time there was debate on the cause and consequence of the disease but in 1876 de Bary demonstrated that the fluffy white stuff was the cause and not the consequence of the infectious disease (DE BARY 1876). Because of its devastating nature he called the infectious agent *Phytophthora infestans* (= infectious plant destroyer). This is the first well-documented report that a plant pathogen caused this amount of damage and this epidemic brought plant pathology into prominence and acceptance. It may not be a coincidence that potato was subject of this drama. Its relatively recent introduction in Europe, started from a limited number of tubers. As a result the genetic basis of potato was also limited. Furthermore potato is generally planted as seed potato and therefore plants are clonal. Consequently, all plants in a field are genetically identical and therefore equally resistant, or in this case, equally vulnerable to pathogen attack. Also plants in neighboring fields must have been genetically highly similar, leading to the further magnification of the epidemic.

Ever since this first outbreak, potato late blight served as a warning for the threat plant diseases pose on food security. This threat is still real in present days. Like in the nineteenth century the staple food of the world population depends on only a few major crops (wheat, rice, maize and potato; FAO 2002). Although the genetic variability in today's crops is much higher than in potato in the nineteenth century the genetic basis of crops is under continuous risk of erosion. Farmers, food processors and consumers have increasing product specifications, which can only be met by one or few genotypes. Global transport may spread infectious diseases over large distances in short times. There is increasing public and political demand for reduction of chemicals that are effective for crop protection but also have side effects, largely neglected in the fifties and sixties. Yet, the prolonged use of chemicals has led to decreased sensitivity to these chemicals by plant pathogens crumpling their effectiveness. *P. infestans* clearly illustrates the complexity of the problems resulting from plant pathogens in agriculture. At the moment, loss of yield and quality and the costs of chemical control of potato late blight alone are around 3 billion US dollars annually (DUNCAN 1999). Despite continuous efforts by farmers, breeders, scientists, and crop protection companies, problems with pathogens persist, and potato late blight is one of the clearest examples of the difficulties plant diseases impose on sustainable food production.

*PHYTOPHTHORA INFESTANS*, THE CAUSAL AGENT OF POTATO LATE BLIGHT.

The disease cycle of *P. infestans* has been well studied (Figure 1). During the growing season, infections usually start from primary infected potato plants with sporangiophores carrying sporangia. These sporangia are wind dispersed and can start

new infections in two ways. Under wet conditions and temperatures below 12 °C, sporangia develop into zoosporangia that release a number of zoospores, each carrying two flagella. After a mobile period, which can last for over ten hours, these zoospores stop moving and a thick cell wall is formed creating a cyst. Alternatively, at higher temperatures sporangia act as sporangiospores that can germinate directly. Both cysts and sporangiospores germinate and at germtube tip an appressorium is formed a specialized structure from which a penetration peg emerges that pierces the cuticle and penetrates the epidermal cell. In the epidermal cell an infection vesicle is formed from which the colonization of the underlying cell layers starts. *P. infestans* grows in between the mesophyll cells where feeding structures (haustoria) are formed. 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. At this time the leaf can still look healthy, without clear symptoms, but more often part of the leaf becomes necrotic and may be surrounded by a white fluffy area where the plant tissue is covered by sporangiophores. *P. infestans* can infect leaves, stems, berries and tubers. While infected tubers are the most common source of inoculum at the beginning of the season (ZWANKHUIZEN et al. 1998), infections can also start from oospores that result from the sexual cycle and can survive several years in the soil (FLIER et al. 2001b). The sexual cycle starts when vegetative hyphae of two opposite mating types (A1 and A2) meet. This induces the formation of oogonia and antheridia. The oogonium grows through the antheridium and after meiosis a fertilization tube grows from the antheridium through the oogonial cell wall and delivers the haploid antheridial nucleus into the oogonium. Subsequently, a thick cell wall is formed making oospores persistent structures. Germinating oospores can form a sporangium, which can start infection of tubers, stems and leaves.

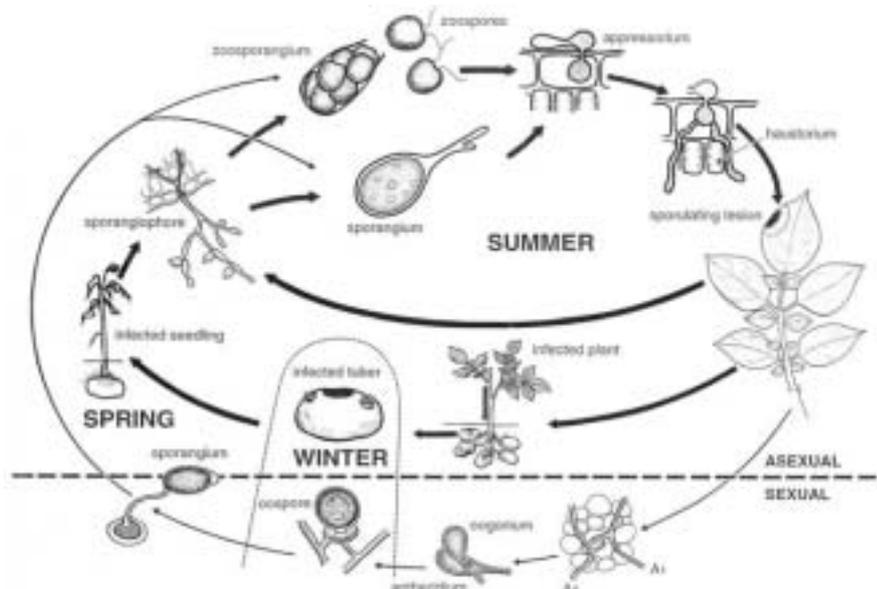


Figure 1: The life cycle of *Phytophthora infestans*

*P. infestans* belongs to the oomycetes. Only in the last decade of the last century it became clear that despite their fungal-like appearance, oomycetes are no fungi, nor are they evolutionary related to fungi. Comparisons of rRNA sequences revealed that oomycetes are more related to chrysophytes and golden-brown algae (FORSTER et al. 1990; VAN DE PEER and DE WACHTER 1997) which are evolutionary as distant from fungi as we humans, are (Figure 2). After many years when scientists tried to reconcile the differences observed between oomycetes and fungi, this brought about a renewed scientific interest for the typical characteristics of oomycetes. These include the presence of mobile zoospores and the distinct chemical composition of the cell wall, which largely consists of cellulose and  $\eta$ -glucans, without chitin (BARTNICKY-GARCIA and WANG 1983). The fact that oomycetes are not related to fungi is particularly relevant for heterologous expression of genes, comparative genomics and genetics in general. Still, despite their different evolutionary origin, the morphology of the hyphae, their mycelium-like growth and the airborne spores, show remarkable resemblance to fungi. Oomycetes and fungi are probably one of the best examples of convergent evolution.

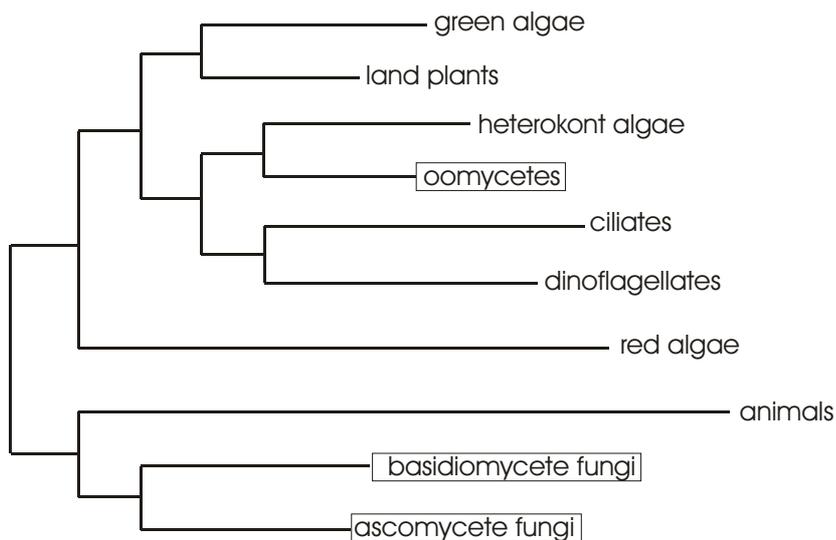


Figure 2: Evolutionary distance between oomycetes and other phylogenetic groups by comparison of rRNA sequences (from KAMOUN et al. 1999 and adapted from VAN DE PEER and DE WACHTER 1997).

The hyphae of oomycetes do not show a clear distinction between the cells and several nuclei can be found in one single cell. Like in fungi the hyphae of different genotypes can fuse resulting in the formation of a single mycelium containing genetically different nuclei called a heterokaryon (VAN WEST et al. 1999). However, unlike fungi, the nuclei of *P. infestans* are diploid and meiosis occurs just before mating in well-differentiated oogonia and antheridia. *P. infestans* is heterothallic with two known mating types, A1 and A2. The haploid number of chromosomes, based on the counts of both meiotic and mitotic

chromosome spreads, is estimated to be between 8 and 10 (SANSOME and BRASIER 1973) and Figure 3.

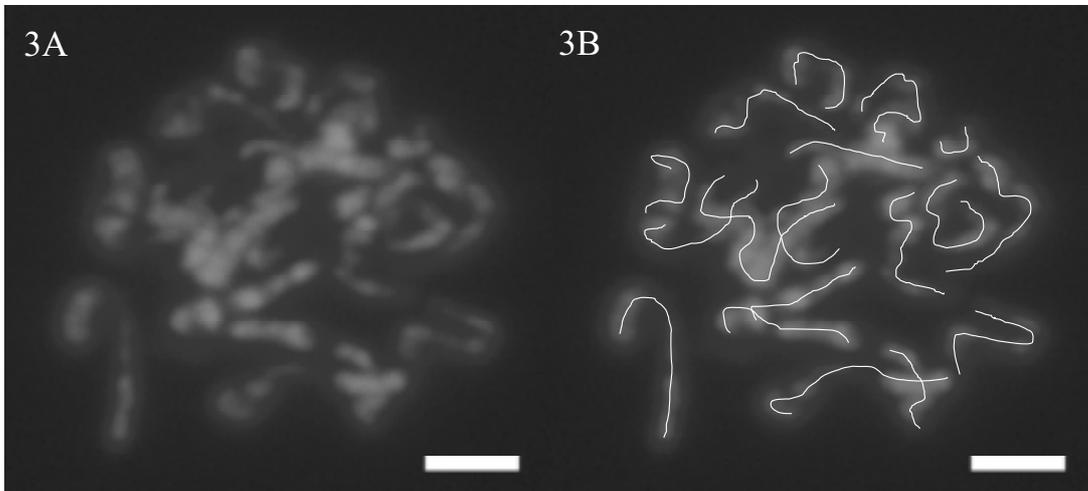


Figure 3: Chromosomes of *Phytophthora infestans* spread by the Germ Tube Burst method and stained with DAPI. Chromosomes are shown without (3A) and with (3B) lines indicating the individual chromosomes. This figure was kindly provided by Masetoki Taga.

#### HOW *PHYTOPHTHORA INFESTANS* COLONISED THE WORLD

Based on the genetic diversity of isolates it was concluded that the centre of origin of *P. infestans* is Mexico (ZENTMYER 1988) and from there this pathogen has colonised the world. Historic data and DNA fingerprinting of preserved samples and isolates allow a reconstruction from the migrations of *P. infestans* over time. Because of the dramatic change it has been speculated that the large *P. infestans* epidemic during the eighteen forties in Western Europe was preceded by an influx of *P. infestans* isolates (ANDRIVON 1996). For a long time many scientists assumed that the *P. infestans* isolates that were found in Europe and the rest of the world outside Mexico between 1848 and the mid-nineteen seventies were direct descendants of this original epidemic. This view was strengthened by DNA fingerprinting showing that all isolates in Europe, North America and the larger parts of Africa and Asia were clonal, represented by a genotype called US-1 (GOODWIN et al. 1994). However, sequencing of historic samples taken from the epidemics of 1846 demonstrated that the *P. infestans* isolates responsible for this epidemic had a different mitochondrial haplotype than the US-1 isolates (RISTAINO et al. 2001). So either the US-1 genotype migrated to Europe later, or this genotype was part of a genetically much diverser set of genotypes of which only the US-1 genotype survived. The US-1 genotype with the A1 mating type dominated the *P. infestans* populations world-wide but in the nineteen seventies in Europe this population was quickly displaced when new *P. infestans* isolates arrived. It has been suggested that these isolates came from Mexico along with the import of large quantities of potato needed after the shortage caused by drought in 1976. With this last migration the genetic diversity increased strongly and the A2 mating type was introduced in

Europe (SPIELMAN et al. 1991). The presence of the two mating types enables the pathogen to reproduce sexually and allows the recombination of traits in the sexual cycle. This generates new possibilities for quick adaptation in the current population in Europe. In general the aggressiveness of *P. infestans* isolates from this last migration seems to be higher and more variable (DAY and SHATTOCK 1997; FLIER and TURKENSTEEN 1999) These new genotypes spread all over Europe and further into Asia and other parts of the world (SMART AND FRY 2001; GHIMIRE et al. 2003). In the USA also new migrations occurred during the last decades of the 20th century. This population probably came from Mexico directly, is genetically less diverse than the population in Europe, but does also contain the two mating types (GOODWIN et al. 1995).

#### METHODS TO CONTROL LATE BLIGHT

To control epidemics of late blight in potato a large set of cultural practices is formulated, which, in conventional agriculture, include the weekly preventive application of chemicals during critical periods in the growing season. Although most scientists agree that it will be difficult to eliminate the dependency on chemical agents for late blight control, for decades breeding for resistant cultivars was postulated as the best option to control *P. infestans* (COLON et al. 1995). However, resistance to *P. infestans* was not found in *Solanum tuberosum*. Therefore breeders and researchers tried to introduce resistance from other *Solanum* species such as *S. demissum*, *S. andigena*, *S. bulbocastanum*, *S. berthaultii*, *S. microdontum* and *S. nigrum* (COLON et al. 1995), so far with limited success. These breeding efforts did not result in commercial cultivars or resistances were not effective in the resulting cultivar due to the variability/adaptation of the pathogen population. At the moment even the most resistant potato cultivars are not sufficiently resistant to prevent late blight epidemics (COLON 2002). Next to this, based on the Dutch national potato list not the most resistant cultivars are grown by farmers (VAN DER WIEL 2002). This is partly due to the fact that in resistance tests performed for the Dutch national potato list only one single *P. infestans* isolate from the seventies is used and resistance to this isolate does not correlate well with resistance to *P. infestans* isolates currently found in the field (FLIER et al. 2001a). In practice farmers do not rely on the resistance levels of potato stated in the Dutch national potato list and the amount of crop protection chemicals used on the different cultivars does not differ much irrespective of their scoring in the list (Personal communication Wilbert Flier, Henk Bonthuis). Another reason why not the most resistant potato cultivars are grown, is that some of the highly susceptible older cultivars, such as Bintje, are still very popular. These cultivars are favored by consumers and food processors because they have become accustomed to the product specifications of these cultivars in a wide range of applications. The old genotypes are also popular by farmers because they generate high yields, are easily marketable and are free from “kwekersrechten”. The latter means that, contrary to modern varieties which can only be grown for specified acreage and can only be sold exclusively to companies for a fixed price, the older varieties can be grown at preferred amounts and sold for the highest price after harvest (Personal communication, Henk Bonthuis). The successful exploitations of new resistance derived from *Solanum* species

remains a challenge for the future and depends largely on the durability of the resistance.

#### THE MECHANISM OF RESISTANCE OF PLANTS TO PATHOGENS

The main reason for the limited success of the introduction of the resistances from other *Solanum* species is that many promising resistance genes failed to work in the commercial cultivar. The resistance genes *R1*, *R2*, *R3*, *R4*, *R5*, *R6*, *R7*, *R8*, *R9*, *R10* and *R11* introgressed from *Solanum demissum* were often already broken before they were introduced in commercial cultivars (TURKENSTEEN 1973). This was remarkable since at the time these resistance genes were introduced only one single clonal lineage of *P. infestans* was present in Europe and America. The speed with which *P. infestans* populations adapted to these new resistance genes is notorious and prompted the view that durable resistance can not be achieved by such single *R*- genes (COLON et al. 1995; PARLEVLIEET 2002). Nevertheless, in natural populations the accumulation of different *R* genes are expected to play an important role to preserve the balance between host and pathogen (SICARD et al. 1999). In this balancing selection, pathogens produce effector proteins that have an added value for viability and/or virulence, but their presence, or activity, can be sensed by the host with the appropriate *R* gene resulting in the effective activation of resistance responses. This is the basis for the gene-for-gene concept (KEEN 1990; KNOGGE 1996; LAUGE and DE WIT 1999). Genes encoding proteins that reveal the presence of the pathogen and trigger effective resistance responses, are called avirulence genes (*Avr* genes). Often this type of resistance is race-specific and the resistance response is associated with a hypersensitive response (HR) resulting in localised death of infected and surrounding cells and in the complete arrest of pathogenic growth. The *R* genes cloned so far can be grouped based on several conserved domains (DANGL and JONES 2001; MCDOWELL and WOFFENDEN 2003). Most of the *R*-genes belong to the NB-LRR, eLRR or LRR-kinase superfamilies, including those for *P. infestans* resistance (BALLVORA et al. 2002; VAN DER VOSSEN et al. 2002). Although the situation is still under debate, many scientists assume that more durable resistance will be achieved by selecting for *R* genes that match with avirulence genes required for virulence, and/or by combined use of different *R*- genes. Still, the disappointments of *R* gene dependent resistance against *P. infestans* experienced in the past are not forgotten and therefore thorough analysis of the genetics and the population dynamics of the loss of *Avr* genes is needed to understand why the *R* genes deployed in the past were not successful. This may help to evaluate the durability of new *R* genes and help to exploit these new resistance genes in resistance management strategies.

#### SCOPE OF THIS THESIS

This thesis describes the results of transmission- and population-genetic studies performed on *P. infestans*, both for the *Avr* genes and neutral markers. After this introduction, which portrays *P. infestans* and the problems associated with it, the second chapter describes the first genetic linkage map generated for *P. infestans*. Linkage analysis was performed in the progeny of a cross between two Dutch field isolates, called

cross 71. We demonstrate that the two parental lines are genetically uniform homokaryons and that the inheritance of markers in their progeny in general follows the laws of Mendel for a diploid organism. Our results show that the mating type is governed by a single locus and that the A1 mating type is dominant. In addition some remarkable features are described such as the distorted segregation ratio for the A1 and A2 mating type in this cross. The mapping of six avirulence genes (*Avr1*, *Avr2*, *Avr3*, *Avr4*, *Avr10* and *Avr11*) is described in chapter three, which also reports the clustering of the avirulence genes *Avr3*, *Avr10* and *Avr11*. High-density genetic linkage maps were generated for the *Avr3-Avr10-Avr11* cluster and for *Avr4*. In all cases avirulence is dominant which fits the gene-for-gene model. Following the genetic mapping we continued with the physical mapping of the avirulence genes (chapter 4). For this we collaborated with Steve Whisson and Paul Birch from the Scottish Crop Research Institute who constructed a BAC library for *P. infestans* isolate T30-4. This is one of the progeny of cross 71 that contains all the segregating avirulence genes in this cross (*Avr1*, *Avr2*, *Avr3*, *Avr4*, *Avr10* and *Avr11*). With the high-density markers surrounding the *Avr3-Avr10-Avr11* cluster a physical contig could be generated and the physical order of the markers in this area could be determined. Chapter five describes the identification of a deletion linked to the *Avr3-Avr10-Avr11* cluster as well as the hemizygous state of this region in the avirulent parent and its absence in the virulent parent. In this respect the parents of cross 71 are not unique: a Mexican isolate that is avirulent on potato carrying the *R3*, *R10* and *R11* resistance gene is also hemizygous for this region. Similarly, in a wide set of genotypes of Dutch field isolates the deletion correlates to virulence on potato plants that carry the *R3*, *R10* and *R11* resistance gene and it is argued that virulence for R genes can be achieved by selection pressure for other R genes. A second generation high-density map for *P. infestans* is presented in chapter six. Detailed genetic analysis identifies a translocation on linkage group III between the parental lines of cross 71 using dominant AFLP markers. The map is compared with the first map (chapter 2) and we accounted for several inconsistencies between the maps. It also shows the alignment of the maps generated by linkage analysis in the progeny of cross 71 and the progeny of cross 68, which suggests several translocations and the presence of trisomic progeny in both crosses. Finally, in chapter seven the implications of the findings described in chapter two to six with emphasis on genetic variability and late blight control are discussed.

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## CHAPTER 2

### AFLP LINKAGE MAP OF THE OOMYCETE

#### *PHYTOPHTHORA INFESTANS*

Fungal Genetics and Biology (1997) **21**: 278-291.



# AFLP Linkage Map of the Oomycete *Phytophthora infestans*

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Van der Lee, T., De Witte, I., Drenth, A., Alfonso, C., and Govers, F. 1997. AFLP linkage map of the oomycete *Phytophthora infestans*. *Fungal Genetics and Biology* **21**, 278–291. Here we present the first comprehensive genetic linkage map of the heterothallic oomycetous plant pathogen *Phytophthora infestans*. The map is based on polymorphic DNA markers generated by the DNA fingerprinting technique AFLP (Vos *et al.*, 1995, *Nucleic Acids Res.* **23**: 4407–4414). AFLP fingerprints were made from single zoospore progeny and 73 F1 progeny from two field isolates of *P. infestans*. The parental isolates appeared to be homokaryotic and diploid, their AFLP patterns were mitotically stable, and segregation ratios in the F1 progeny were largely Mendelian. In addition to 183 AFLP markers, 7 RFLP markers and the mating type locus were mapped. The linkage map comprises 10 major and 7 minor linkage groups covering a total of 827 cM. The major linkage groups are composed of markers derived from both parents, whereas the minor linkage groups contain markers from either the A1 or the A2 mating type parent. Non-Mendelian segregation ratios were found for the mating type locus and for 13 AFLP markers, all

of which are located on the same linkage group as the mating type locus. © 1997 Academic Press

**Index Descriptors:** *Phytophthora infestans*; oomycete; AFLP; linkage map; plant pathogen; potato late blight; AFLP DNA fingerprinting; fungi; mating type; non-Mendelian segregation; JOINMAP.

In the past decade genetic linkage maps of many organisms have been generated. These maps are essential for positional cloning of genes which are linked to particular phenotypes. Genetic linkage maps are also useful for other purposes, for example, to gain insight into evolutionary relationships between organisms or to analyze genome rearrangements, events which frequently occur in organisms with flexible genomes such as fungi (Caten, 1996; Mills and McCluskey, 1990).

The explosive development in mapping is mainly due to the availability of various types of molecular markers, of which inheritance is easy to score. In 1980 Botstein *et al.* described a new basis for the construction of genetic linkage maps of complex genomes. Restriction fragment length polymorphisms (RFLPs) detected by hybridization of genomic DNA with anonymous DNA probes were introduced as markers for genetic linkage analysis. A few years later the first RFLP map of the human genome which covered all 23 chromosomes was published (Donis-Keller *et al.*, 1987). Since the discovery of the polymerase chain reaction (PCR) in the mid-eighties (Mullis *et al.*, 1986), many different types of DNA markers have been developed. Most of these are based on amplification of

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random genomic DNA fragments using arbitrarily selected PCR primers and low annealing temperatures to allow the primers to anneal to multiple loci on the DNA. This results in complex DNA fingerprinting patterns and many different markers can be scored in one experiment. This is in contrast to RFLP mapping, where in most cases only one or a few loci are detectable in one experiment. Further improvement of PCR-based DNA fingerprinting technology was accomplished by Vos *et al.* (1995), who developed a technique called AFLP DNA fingerprinting. The advantage of AFLP DNA fingerprinting above other PCR-based DNA fingerprinting methods is that it is more reliable and more reproducible. Prior to amplification, adapters with known sequences are ligated to digested genomic DNA, and for PCR, primers which perfectly match the adapter sequences are used, allowing stringent annealing conditions. In a second PCR labeled primers which are extended with one, two, or three selective bases at the 3' end are used. In this way the number of fragments that will be amplified simultaneously can be chosen in the range from 10 to 100.

In the present study we exploit the AFLP fingerprint technology to obtain a large number of markers for constructing a genetic linkage map of the plant pathogen *Phytophthora infestans*. *P. infestans* is the causal agent of potato late blight and belongs to the oomycetes, a group of diverse organisms that include significant pathogens of plants, insects, and animals as well as saprophytic species. Despite the fact that oomycetes exhibit a fungal-like growth morphology they are no longer classified as members of the Kingdom Fungi. Phylogenetic studies based on rDNA sequences revealed that oomycetes evolved along a evolutionary line different from that of ascomycetes and basidiomycetes (Förster *et al.*, 1990). Oomycetes are more closely related to brown algae and diatoms, and in recent classification schemes the phylum Oomycota is placed in the Kingdom Chromista (Hawksworth *et al.*, 1995; Erwin and Ribeiro, 1996). The somatic hyphae of oomycetes are diploid. Meiosis occurs just before mating in well-differentiated oogonia (♀) and antheridia (♂). Some species are homothallic whereas others, including *P. infestans*, are heterothallic. The two known mating types in the genus *Phytophthora* have been designated A1 and A2.

For a long time genetic studies on *Phytophthora* species and other oomycetes were hampered (reviewed by Judelson, 1996) by the lack of morphological and biochemical mutants which have been very useful in unravelling the genetics of true fungi. Instead isozyme markers were used, but again this approach faced limitations because very few polymorphisms were found. In *P. infestans* only 3 of 50

tested enzymes appeared to be polymorphic (Tooley *et al.*, 1985). When DNA markers were developed, inheritance studies in oomycetes could be performed in a more efficient way. So far, genetic linkage maps of two oomycetous plant pathogens have been published: a preliminary RFLP map of the lettuce downy mildew pathogen *Bremia lactucae* (Hulbert *et al.*, 1988) and a map of the root and stem rot pathogen *Phytophthora sojae* consisting of RFLP and RAPD (random amplified polymorphic DNAs) markers (Whisson *et al.*, 1995). In *P. infestans* genetic mapping has been limited to a restricted region of the genome. Judelson *et al.* (1995) selected several polymorphic DNA markers that are linked to the mating type locus and analyzed the inheritance in three different crosses. In that way they constructed a map of a region covering approximately 100 kb. Also Goodwin *et al.* (1992) analyzed inheritance of polymorphic DNA markers in sexual progenies of *P. infestans* and found some linked markers. However, the latter study was not aimed at mapping but rather at evaluating the use of RFLP markers detectable with moderately repetitive DNA fingerprint probes for population genetic studies (Goodwin *et al.*, 1992).

The aims of this study were (i) to develop an efficient AFLP DNA fingerprinting protocol for *P. infestans*; (ii) to use AFLP markers for analyzing the genetic heterogeneity in single zoospore cultures of *P. infestans*; (iii) to analyze the segregation of AFLP markers in a sexual progeny of *P. infestans*; and (iv) to construct a genetic linkage map of *P. infestans*.

## MATERIALS AND METHODS

### *Phytophthora infestans* Mapping Population

The mapping population is an F1 progeny from two Dutch *P. infestans* isolates, 80029 (A1 mating type) and 88133 (A2 mating type), obtained in two independent crosses (collectively called cross 71). The initial characterization is described by Goodwin *et al.* (1992) and Drenth *et al.* (1995). Part of the progeny is derived from oospores generated *in vitro* on V-8 agar and the other part from oospores generated in infected leaves (*in vivo*). The *in vitro* progeny was recovered by culturing single germinating oospores. The *in vivo* progeny was recovered from sporulating lesions formed on leaves that were floated on water containing soil with oospores. The latter procedure allowed recovery of more than one F1 progeny from a single oospore.

## Culturing of *Phytophthora infestans* and Generation of Single Zoospore Isolates

The *P. infestans* isolates 80029 and 88133 and their F1 progeny were maintained on rye-agar medium containing 2% sucrose (Caten and Jinks, 1968). The mating type of the F1 progeny was determined by growing strains on clarified rye-agar medium adjacent to strains with known mating type (A1 strain 88069 or A2 strain 90128). After 7–14 days the cultures were checked for the presence of oospores.

Single zoospore isolates were obtained according to the method of Caten and Jinks (1968) with some modifications. Sporulating mycelium was obtained by culturing isolates 80029 and 88133 on rye-agar for 2 weeks at 18°C in the dark. The mycelium was covered with 10 ml sterilized tap water and incubated at 4°C for 3 h to allow zoospores to be released from the sporangiospores. The zoospore suspension was removed and diluted to a concentration of 100 zoospores per milliliter. Each well in a 96-well microtiter plate was filled with 60 µl modified Plich medium (containing per liter 0.5 g KH<sub>2</sub>PO<sub>4</sub>, 0.25 g MgSO<sub>4</sub> · 7H<sub>2</sub>O, 1 g asparagine, 1 mg thiamine, 0.5 g yeast extract, 10 mg β-sitosterol, 25 g glucose, and 15 g agar) and 10 µl of the zoospore suspension. The plates were incubated at 18°C. Every day growth was checked microscopically and wells containing only one germinating cyst were marked. After a few days, when hyphal growth was observed, the contents of marked wells were transferred to Petri dishes containing rye-agar medium and the single zoospore cultures were cultured as described above.

## DNA Isolation

*P. infestans* mycelium was cultured for 2 weeks in 9-cm Petri dishes containing 5 ml liquid rye medium. From the mycelium DNA was isolated according to Drenth and Govers (1993). The procedure was adapted to a small scale starting with 10–20 mg lyophilized mycelium that was ground with an equal amount of sand (SiO) and mixed with 1 ml extraction buffer and 500 µl phenol.

## AFLP DNA Fingerprinting

AFLP DNA fingerprinting was performed essentially as described by Vos *et al.* (1995). The AFLP protocol consists of four steps: (i) primary template preparation in a one-step restriction-ligation reaction in which adapters are ligated to restriction fragments of genomic DNA; (ii) selective preamplification of primary template with primers complementary to the adapters and containing one 3'

selective nucleotide; (iii) selective amplification with the same primers but now radioactively labeled and containing two 3' selective nucleotides; and (iv) separation of the labeled fragments on polyacrylamide gels followed by autoradiography.

**Template preparation.** Primary template DNA was prepared by incubating 250 ng genomic DNA for 4 h at 37°C with 5 U *Eco*RI, 5 U *Mse*I, 5 pmol *Eco*RI adapter, 50 pmol *Mse*I adapter, 0.2 mM ATP, and 1 U T4 DNA-ligase in 50 µl restriction-ligase buffer (10 mM Tris-HAc, pH 7.5, 10 mM MgAc, 50 mM KAc, 5 mM DTT, 50 ng/µl BSA). The sequence of the adapter fitting the *Eco*RI site was

5'-CTCGTAGACTGCGTACC  
CTGACGCATGGTTAA-5'.

The sequence of the adapter fitting the *Mse*I site was

5'-GACGATGAGTCCTGAG  
TACTCAGGACTCAT-5'.

Adapters were prepared by adding equimolar amounts of both strands. The adapters were not phosphorylated. After the restriction-ligation reaction, the reaction mixture was diluted to 500 µl with 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0, and stored at -20°C.

**Selective preamplification of primary template DNA.** The purpose of this first selective PCR amplification is to reduce the complexity of the template fragments and to generate large quantities of template suitable for the second PCR amplification with radioactively labeled selective primers. The primers used in the preamplification step are so-called E+1 and M+1 primers that correspond to the *Eco*RI and *Mse*I ends of the primary template, respectively. "+1" indicates that the primer contains one selective nucleotide. The sequence of the E+1 primer was 5'-GACTGCGTACCAATTCA (E+A), in which A is the selective nucleotide, whereas the M+1 primers had the sequence 5'-GATGAGTCCTGAGTAAA (M+A) and ATGAGTCCTGAGTAAC (M+C). Five microliters of the diluted primary template DNA was mixed with 30 ng E+A primer, 30 ng M+A or M+C primer, 0.2 mM dNTPs, and 0.7 U *Taq* DNA polymerase (Perkin-Elmer, Norwalk, CT) in 10 µl 10 mM Tris, pH 8.3, 1.5 mM MgCl<sub>2</sub>, and 50 mM KCl. Amplification was accomplished by PCR in a PE-9600 thermal cycler (Perkin-Elmer). The PCR started with a cycle of 30s at 94°C, 30 s at 65°C, and 1 min at 72°C and was followed by 13 cycles with a stepwise lowering of the annealing temperature by 0.7°C in each cycle (touchdown profile). After the preamplification the reaction mixture was diluted to 200 µl with 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0, and stored at -20°C.

in repulsion phase with the mating type locus are underrepresented. This confirms that mating type involves a single locus that is heterozygous in the A1 parent with A1 dominant ( $Aa \times aa$ ) (Shaw, 1991; Judelson *et al.*, 1995). The observation that mating type does not segregate according to Mendelian ratios has also been reported by others. Judelson *et al.* (1995) analyzed segregation of the mating type locus and polymorphic DNA markers linked to that locus. They found in two progenies more A1 phenotypes than expected. Moreover, from the markers linked to the mating type locus they found only two of the four expected genotypes in the progeny. These findings are reminiscent of the occurrence of balanced lethality, and Judelson *et al.* (1995) proposed a genetic model to explain the absence of certain marker combinations. However, with our data we cannot support this model. Either balanced lethality did not occur in cross 71 or the markers we mapped on LG III are too far away from the mating type locus to allow detection of this phenomenon in the cross 71 progeny.

Many previous studies on *P. infestans* reported spontaneous variability found in field or laboratory isolates (Caten, 1970; Caten and Jinks, 1968; Denward, 1970; Le Grand-Pernot, 1986, 1988). Most of these authors suggested that the frequent appearance of new races in the field that could overcome major resistance (R) genes introgressed in potato for late blight resistance, was attributed to the heterokaryotic nature of the parental isolate. Unfortunately that hypothesis was not testable because genetic differences between the various single zoospore isolates could not be traced. With AFLP DNA fingerprinting many independent loci in the genome can be analyzed in one experiment. Therefore this technique might be helpful in readdressing the question of variability in *P. infestans*. In this study we analyzed single zoospore progenies of two field isolates. Despite the fact that we found variability in colony morphology, growth rate, and spore production in the single zoospore isolates we did not find differences in AFLP DNA fingerprinting patterns. Clearly the parental isolates we used were not heterokaryons. The variability might be caused by minor changes in the genome like point mutations or deletions but also methylation, transposable elements, or cytoplasmic dsRNA might play a role. Retrotransposon-like elements and dsRNA molecules have been found in *P. infestans* (Tooley and Garfinkel, 1996; Newhouse *et al.*, 1992) but whether they are active or related to a certain phenotype remains to be investigated.

*P. infestans* is a major pathogen of potato. Our aim is to unravel the molecular and genetic basis of race specificity in the potato-*P. infestans* pathosystem. By resistance

breeding 11 dominant R genes from wild *Solanum* species have been introduced into the cultivated potato. To isolate the *P. infestans* genes whose products specifically interact with R-gene products we follow a map-based cloning approach. The AFLP linkage map presented here forms the basis for this approach. Determination of virulence and avirulence characteristics of part of the F1 progeny on a differential set of potato lines carrying major R genes has shown that in cross 71 six relevant genes segregate (Alfonso and Govers, 1995). Virulence data of more F1 progeny are required to place the genes reliably on the map. Simultaneously, the map will be saturated with additional AFLP markers and other crosses with segregating virulence characteristics will be mapped. We expect that with the help of a genetic linkage map of *P. infestans* and the availability of the AFLP marker technology we will be able to analyze the inheritance of (a)virulence phenotypes in *P. infestans* and to clone avirulence genes.

## ACKNOWLEDGMENTS

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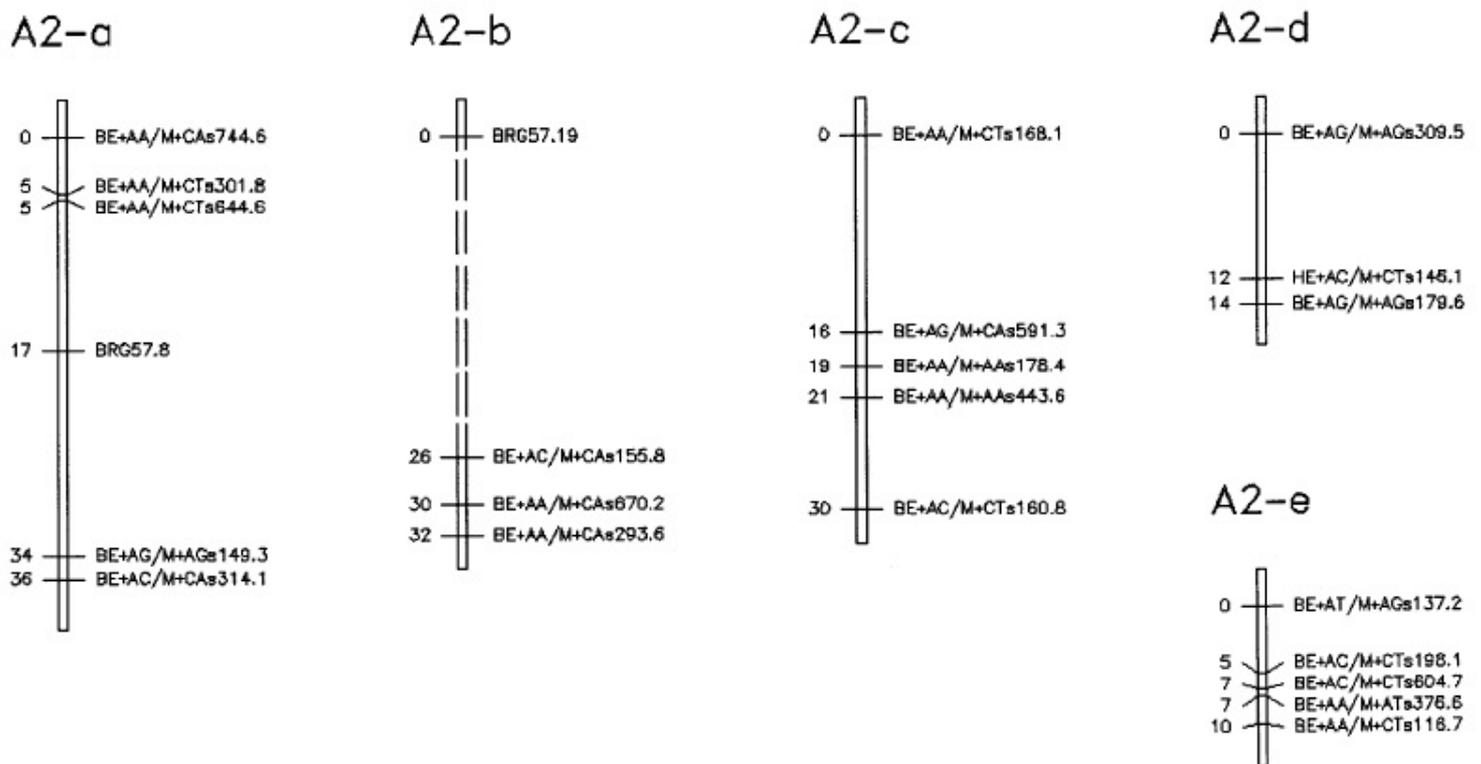


FIGURE 4.—Continued

*et al.*, 1988), varied between 1010 and 1340 cM. We estimate the total genome size to be ca. 1200 cM. With a physical size of 250 Mb, 1 cM relates to approximately 200 kb. Of the 1200 cM, 624 cM are mapped between markers on the integrated linkage groups I to X, whereas 81 and 122 cM are covered by the two A1 and five A2 linkage groups, respectively. Since the A1 and A2 linkage groups might cover overlapping regions, we cannot exactly determine how many centimorgans are currently mapped. But even if we sum up the centimorgans to 827 in total, ca. 69% of the genome is covered between markers despite the fact that nearly 90% of the markers were mapped. Although there is a rather even distribution of markers over the various linkage groups, there are some regions where markers are underrepresented. Whether the low marker density of these regions is related to high recombination frequencies or coincides with low polymorphism between the parental isolates must be determined by mapping other crosses.

Most AFLP markers segregated according to Mendelian ratios. Only 13 AFLP markers showed clear distorted segregation ratios. These 13 markers all originate from the A1 parent and map on LG III, the linkage group that contains the mating type locus. In fact, all A markers on LG III segregate aberrantly. A markers in linkage phase with the mating type locus are, like the A1 mating type itself, strongly overrepresented in the progeny, whereas markers

TABLE 2

Linkage Groups of *Phytophthora infestans*

Linkage group	Length (cM)	Number of markers	Average spacing <sup>a</sup> (cM)	Largest interval <sup>b</sup> (cM)
I	91	26	3.6	11
II	90	21	4.5	26
III	81	28	3	12
IV	73	20	3.8	15
V	67	15	4.8	28
VI	66	13	5.5	19
VII	57	8	8.1	13
VIII	54	9	6.3	18
IX	40	12	3.6	10
X	5	6	1	2
A1-a	58	5	14.5	26
A1-b	23	5	5.8	8
A2-a	36	6	7.2	17
A2-b	32	4	10.7	26
A2-c	30	5	7.5	16
A2-d	14	3	7	12
A2-e	10	5	2.5	5
	827 <sup>c</sup>	191 <sup>c</sup>	4.3 <sup>d</sup>	

<sup>a</sup> Average spacing between markers.

<sup>b</sup> Largest interval between two markers.

<sup>c</sup> Total of all linkage groups.

<sup>d</sup> Average for all linkage groups.

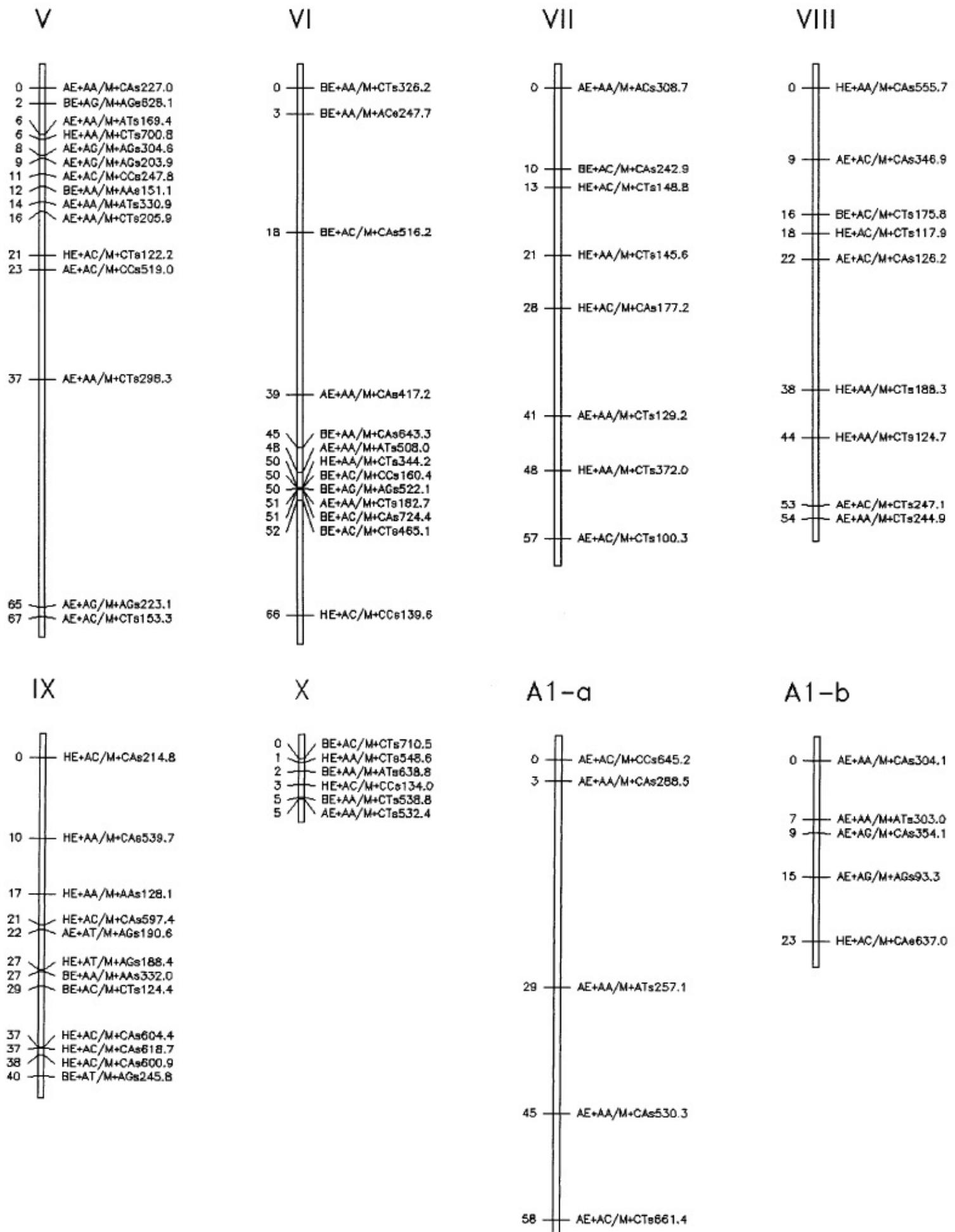


FIGURE 4—Continued



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dashed line on A2-b in Fig. 4) linkage could only be obtained by using LOD threshold values lower than 3 (LOD = 2.5).

Of the 217 scored markers 25 AFLP markers and 1 RFLP marker (listed in the legend to Fig. 4) were not positioned on the map. Twelve of these showed no linkage to at least two other markers. The others were linked, but positioning of these markers on the map caused such a high increase in the  $\chi^2$  value that it decreased the reliability of the map. Nevertheless, all the unlinked markers segregated according to Mendelian segregation ratios. For 13 AFLP markers as well as for the mating type locus non-Mendelian segregation ratios were found. In the F1 progeny those markers are either underrepresented (classes 10–15, 16–21, and 22–27 in Fig. 3) or overrepresented (classes 58–63 and 64–69) and they do not fit within the theoretically calculated curve, which reflects a 1:1 segregation (Fig. 3). All these markers (indicated by asterisks in Fig. 4) map on one linkage group, LG III. The 13 aberrantly segregating AFLP markers are all A markers. None of the B markers located on LG III showed significant deviation from the expected Mendelian segregation ratios. For the H markers nonMendelian segregation is only detectable when heterozygosity can be discriminated from homozygosity. Since the three H markers located on LG III were scored for presence (A\*) and absence (aa), it could not be determined whether they segregated aberrantly.

The total genome coverage represented by all linkage groups is 827 cM. It should be noted, however, that an A1 linkage group might actually represent the same genomic region as an A2 linkage group and therefore the mapped distance cannot be determined exactly. By calculating the average distance between markers for every linkage group separately, we determined that in the mapped region we obtained an average of one marker for every 4.3 cM. Based on the method presented by Hulbert *et al.* (1988), we calculated the genome size of *P. infestans*. For the A1 parent 80029 we obtained genome sizes of 1053 cM and 1010 cM with LOD threshold values of 3 and 4, respectively. For the A2 parent 88133 slightly larger sizes were found, 1324 cM and 1340 cM for LOD 3 and 4, respectively.

## DISCUSSION

In this report we have presented the first detailed genetic linkage map for the heterothallic oomycetous plant pathogen *Phytophthora infestans*. The map is based on polymorphic DNA markers generated by a powerful DNA

fingerprinting technique called AFLP (Vos *et al.*, 1995). The AFLP DNA fingerprinting protocol was optimized for *P. infestans*. Small adjustments were made mainly to cope with the relatively large fragments obtained after digestion of *P. infestans* DNA with *EcoRI* and *MseI*. With the adjusted protocol AFLP fingerprints were made from single zoospore progenies and F1 progenies (cross 71) from two field isolates of *P. infestans*. The parental isolates appeared to be homokaryotic and diploid, their AFLP patterns were mitotically stable, and segregation ratios in their F1 progeny were mostly Mendelian. Therefore cross 71 could be used for constructing a genetic linkage map.

The map consists of 190 polymorphic DNA markers and one phenotypic marker distributed over 17 linkage groups. Ten major linkage groups, LG I–X, are composed of A, B, and H markers derived from the two parental isolates. The remaining seven are minor A1 and A2 linkage groups with either A or B markers. Combinations of A1 and A2 linkage groups might actually represent additional major linkage groups but as long as no H markers are found that can form the bridge they cannot be integrated. Additionally, minor linkage groups might be linked to a major linkage group or might form the link between two other linkage groups. To find this linkage and to be able to incorporate in the map markers that were unlinked or linked to only one other marker, we must increase the number of markers or, as an alternative, enlarge the mapping population. One of the advantages of AFLP DNA fingerprinting is that many markers can be scored in one experiment. In the cross 71 mapping population on average 19 AFLP markers were scored per primer combination and 89% of these markers could be located on the map. The number of possible E+2/M+2 primer combinations is 256. Hence about 4500 more markers can be generated with just the *EcoRI/MseI* enzyme combination.

The major linkage groups I–X which contain markers from both parents might represent chromosomes of *P. infestans*. Unfortunately, the exact chromosome number in *P. infestans* is still unknown. Cytological studies performed in the early seventies by Sansome and Brasier (1973) revealed that a gametangium of *P. infestans* in the meiotic prophase contains 16–20 chromosomes, indicating that the haploid chromosome number is  $9 \pm 1$ . The small sized chromosomes of *P. infestans* are difficult to resolve by light microscopy, which makes accurate counting impossible. Also, attempts to determine the number of chromosomes of *P. infestans* by pulsed field gel electrophoresis (PFGE) were unsuccessful (Tooley and Carras, 1992). Unlike chromosomes of six other *Phytophthora* species, chromo-

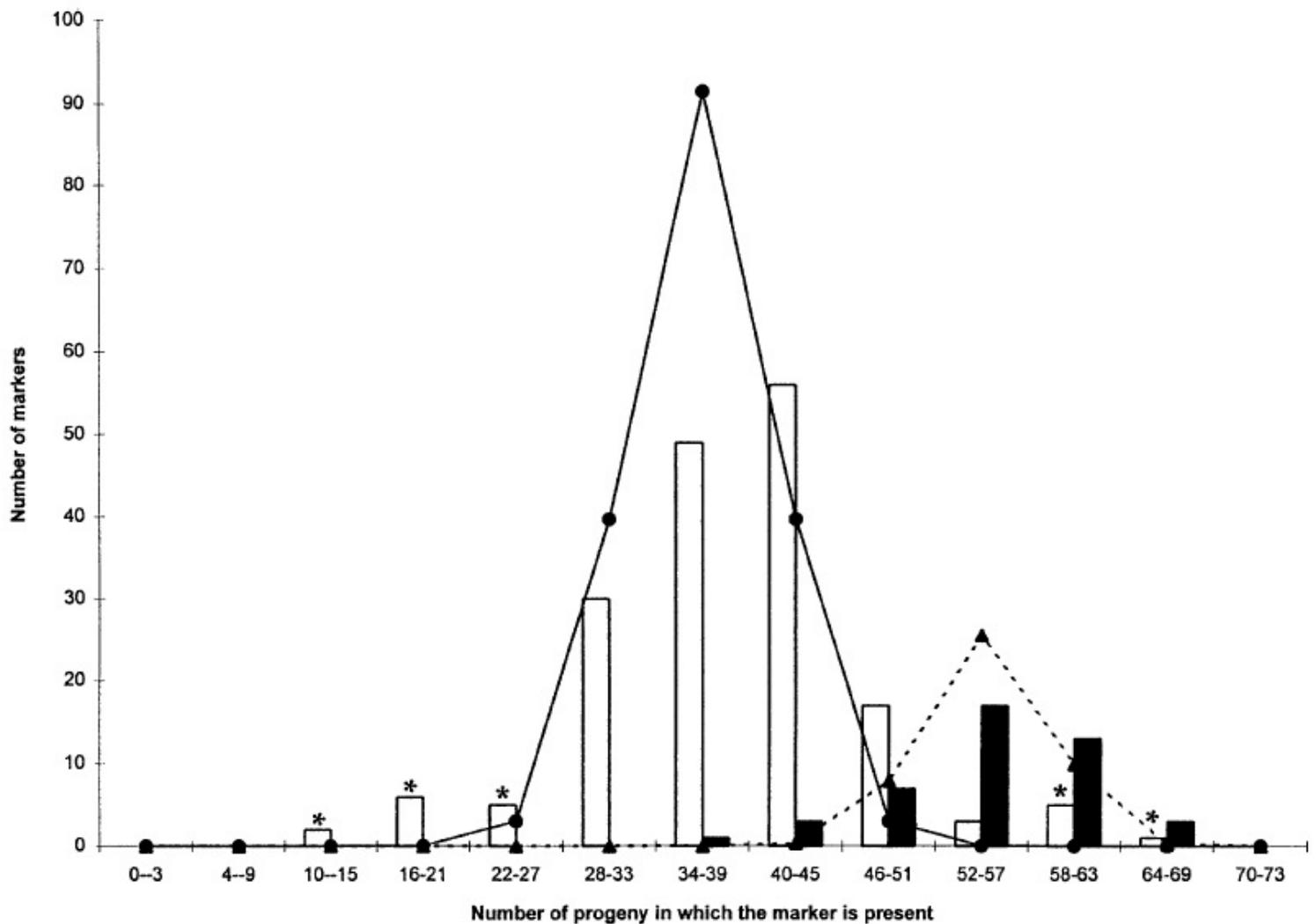


FIG. 3. Segregation ratios of AFLP markers in 73 F1 progeny of *P. infestans* cross 71. The number of F1 progeny in which an AFLP marker was found was divided into 13 classes (X-axis). The number of AFLP markers (Y-axis) in each class is shown by bars. Open bars represent A and B markers; solid bars represent H markers. In case segregation of a marker was determined in less than 73 F1 progeny the ratio was adjusted to  $N = 73$ . The distribution of presence and absence was compared with the expected binomial distribution (curve solid line, A and B markers; curve dashed line, H markers). The theoretically expected ratios were calculated with a probability of presence in the progeny of 0.5 for A and B markers, 0.75 for H markers, and  $N = 73$ . Bars marked with an asterisk indicate the classes containing aberrantly segregating markers located on LG III.

and 3. For every LOD threshold value a map was calculated and the stability was checked upon addition of new markers. At a LOD threshold value of 3 the order of markers in some linkage groups changed dramatically, indicating erroneous linkage. We therefore continued with a LOD threshold value of 4. At this LOD value 15 linkage groups consisting of at least three markers were identified with the A1 data set and 17 with the A2 data set (data not shown).

In the next step the two most distal markers from each linkage group were collected in a new data set together with markers that were not yet positioned on one of the linkage groups and markers that were linked to only one other marker. Subsequently, linkage was determined at a LOD threshold value of 3 and 2.5, and this reduced the

number of A1 linkage groups to 12 and the number of A2 linkage groups to 15. In addition, several previously unlinked markers could be integrated.

Finally an integrated map was constructed by using the H markers present in both the A1 and the A2 map as bridges. The order of the A and B markers was fixed according to the A1 and A2 map, as in the integrated map an A marker should not influence the position of a B marker and vice versa. As shown in Fig. 4 and summarized in Table 2, the map contains in total 191 markers distributed over 10 integrated linkage groups (LG I to X with A, B, and H markers) and 7 minor linkage groups which contain either A (A1-a and A1-b) or B markers (A2-a to A2-e) and some H markers. In one region (indicated by a

tions were selected and the genetic stability of the parental lines 80029 and 88133 was determined. Of 32 E+2/M+2 primer combinations tested on the parental isolates, 11 that produced highly informative DNA fingerprinting patterns were selected. On average 10% of the amplified fragments found in one parent was not present in the other parent and vice versa. Of these, only the heterozygous fragments are informative for mapping. Also amplified fragments present in both parents (90%) can be informative markers when they are heterozygous in both parents (Table 1). In the F1 progeny of cross 71 approximately one-fifth of the total number of AFLP bands detected in fingerprints of either 80029 or 88133 segregated (see below), and thus the level of heterozygosity was estimated to be 20% in both parental lines.

The F1 progeny of cross 71 consists of individuals derived from two separate crossing experiments performed under different conditions and in different years (see Materials and Methods). To investigate the genetic stability of the parental lines we analyzed AFLP fingerprints of several DNA samples of 80029 and 88133 isolated at different time points and from different batches of mycelium. The patterns obtained with two primer combinations were the same for all 80029 DNA samples and all 88133 DNA samples (data not shown).

To be able to compare the nuclei present in the coenocytic mycelium of the parental isolates 80029 and 88133, single zoospore cultures were made. In this way it is possible to check whether the parental isolates are homokaryons or heterokaryons. Since single zoospores presumably contain only one nucleus, all single zoospore cultures of a homokaryon should have the same AFLP fingerprint pattern identical to that of the parental line. AFLP patterns of the single zoospore cultures obtained with two primer combinations and comprising in total 177 different amplified fragments were compared and the data were used for genetic clustering analysis with NTSYS. The patterns of 12 single zoospore isolates of 80029 and 21 single zoospore isolates of 88133 were identical to those of 80029 or 88133, respectively (Fig. 1). One isolate, 80029-4, had the same AFLP fragments as the parental isolate 80029 but there was one additional fragment which was not found in 80029 nor in the other single zoospore isolates. This was the only aberrant fragment found among 177 fragments in 34 single zoospore isolates and it was never found in fingerprints obtained with various DNA samples of the parental line 80029. Moreover, the difference between AFLP fingerprints of 80029, 88133, and 40 nonrelated *P. infestans* isolates (Fig. 1 and data not shown)

is much larger than the difference between 80029 and 80029-4. Therefore, it is likely that the appearance of this unique fragment is an artifact or is due to a rarely occurring event, such as a point mutation or deletion, rather than to heterogenous nuclei in the parental isolate. Hence, we conclude that 80029 and 88133 are homokaryons and that the AFLP patterns are mitotically stable.

### *AFLP Analysis of the F1 Progeny of Cross 71*

From the cross 71 F1 progeny 77 individuals were subjected to AFLP analysis. As shown in Fig. 2 all the possible inheritance patterns (shown schematically in Table 1) were found. From the 208 segregating AFLP markers that were scored, 79 were type 6 or A markers, 85 were type 8 or B markers, and 44 were type 5 or H markers. For most H markers intensity differences were clear enough to allow differentiation between AA and Aa.

The segregation ratios found for A, B, and H markers in cross 71 were compared to the ratios expected when segregation of markers in an F1 progeny occurs in a Mendelian fashion. Theoretically all three marker types should have a binomial distribution. For A and B markers the probability of presence in the progeny is 0.5 and for H markers, if scored for presence or absence, 0.75. The distribution of the segregation ratios found in F1 progeny of cross 71 (shown in bars in Fig. 3) clearly resembles the expected distribution (shown in curves in Fig. 3). Thirteen markers that segregate aberrantly, outside the theoretically calculated curves (bars indicated by asterisks in Fig. 3), all map on the same linkage group, i.e., LG III (see below). We conclude that in this cross most molecular markers segregate in Mendelian ratios, thus confirming that the parental lines 80029 and 88133 are diploid.

### *Linkage Analysis and Map Construction*

In total 208 AFLP markers, 8 RFLP markers, and the mating type were scored in 77 F1 progeny. To check for genotypically identical F1 individuals that might be derived from one oospore, genetic cluster analysis was performed. Seventy-three unique genotypes were found so four F1 individuals were excluded from further analysis. With data obtained from the remaining 73 F1 individuals linkage analysis was performed using the software program JOINMAP. Initially two maps were created, one based on the A1 data set and the other on the A2 data set. Linkage was determined at LOD threshold values of 8, 7, 6, 5, 4,

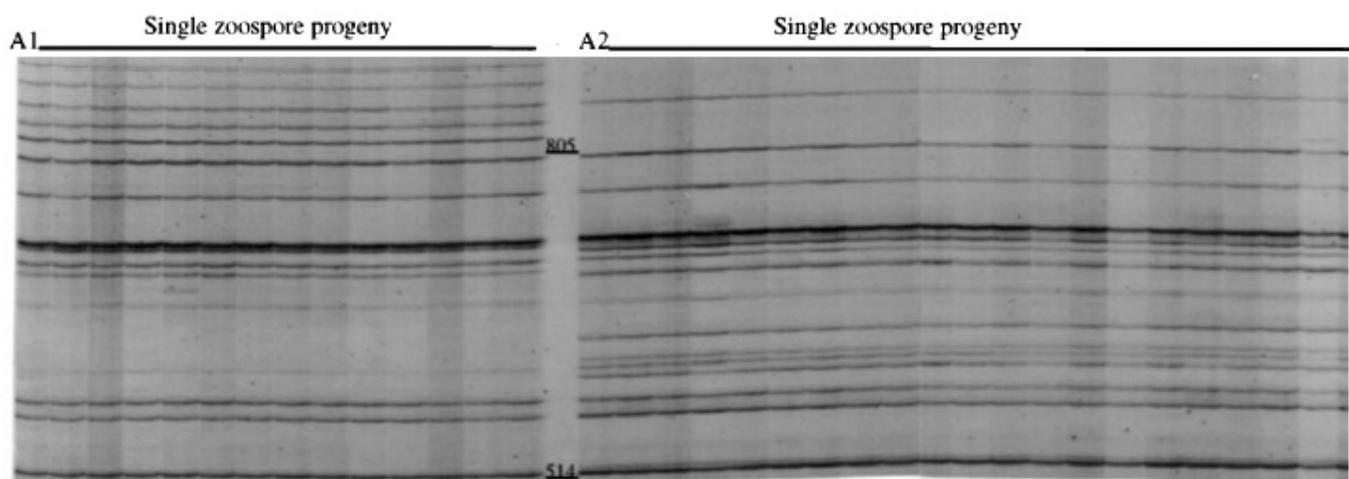


FIG. 1. Section of autoradiograph showing AFLP DNA fingerprints of *P. infestans* isolate 80029 (indicated by A1) followed by asexual single zoospore progeny of 80029 (13 lanes) and isolate 88133 (A2) followed by asexual single zoospore progeny of 80133 (20 lanes). The AFLP DNA fingerprints were generated with the primer combination E+AG/M+CA. DNA size markers (805 and 514) are from *Pst*I-digested  $\lambda$  DNA. Approximately 20% of the total length of the lanes is shown.

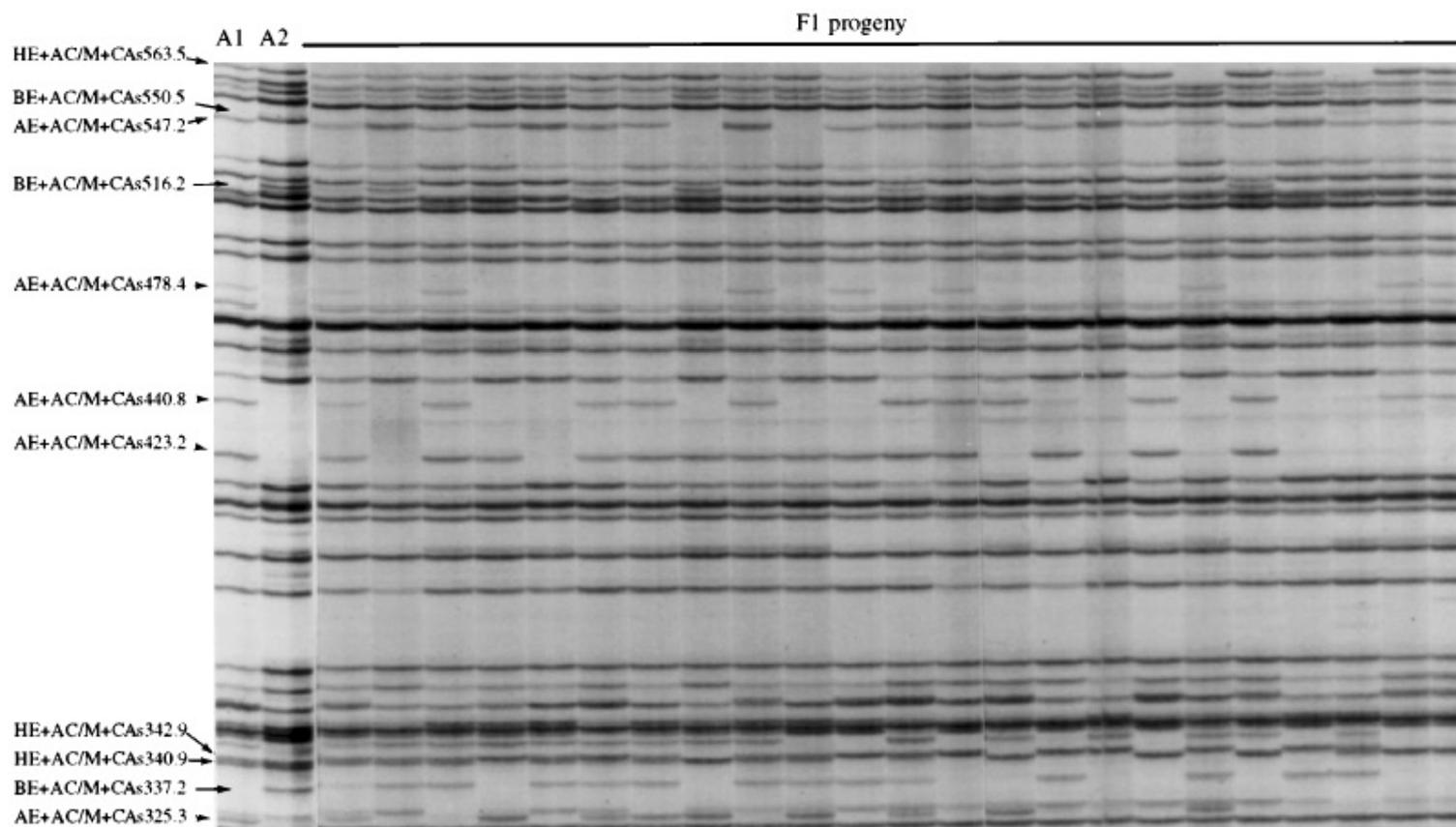


FIG. 2. Section of autoradiograph showing AFLP DNA fingerprints of *P. infestans* A1 isolate 80029 (A1), A2 isolate 88133 (A2), and 24 of their sexual F1 progeny (F1 progeny). The AFLP DNA fingerprints were generated with the primer combination E+AC/M+CA. Markers segregating in the F1 progeny are indicated on the left. For nomenclature of AFLP markers see text. Approximately 20% of the total length of the lanes is shown.

TABLE 1  
Inheritance of Amplified Fragments in the F1 Progeny of Diploid Organisms

Genetic model	Expected ratio	AFLP phenotypes																	
		Parents						Offspring											
1. AA × AA → AA	1:0	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
2. AA × Aa → AA, Aa	1:1	■	-	■	-	■	-	■	-	■	-	■	-	■	-	■	-	■	-
3. AA × aa → Aa	1:0	■	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4. Aa × AA → AA, Aa	1:1	-	■	-	-	■	■	-	-	■	-	■	-	■	-	■	-	■	-
5. Aa × Aa → AA, Aa, aa <sup>a</sup>	1:2:1	-	-	-	-	-	-	-	-	■	-	■	-	-	-	■	-	■	-
6. Aa × aa → Aa, aa <sup>a</sup>	1:1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7. aa × AA → Aa	1:0	■	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8. aa × Aa → Aa, aa <sup>a</sup>	1:1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9. aa × aa → aa	0:1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

<sup>a</sup> Type 5 markers have a prefix H; type 6 markers have a prefix A; and type 8 markers have a prefix B.

The data files with segregating markers were used as input files for the software program JOINMAP, Version 1.4 (Stam, 1993). JOINMAP first establishes linkage of markers by calculation of the Logarithm of Odds (LOD) value for each marker pair and by comparing this to the threshold LOD value. Subsequently the linear order of markers within a linkage group is calculated. Initially two separate parental maps were constructed. The A1 map was composed from a data set containing A and H markers and the A2 map from a data set containing B and H markers. With each data set linkage groups were identified by stepwise lowering of the LOD threshold value from 8 to 3. Within each linkage group the linear order of the markers was determined at a LOD threshold value of 0.05 and with the Kosambi mapping function. To join the two maps into one combined map JOINMAP uses the H markers as bridges. Output from JOINMAP was converted to figures using the companion graphics program DRAWMAP (Van Ooijen, 1994).

## RESULTS

### An Efficient AFLP DNA Fingerprinting Protocol for *P. infestans*

The AFLP technique generates DNA fingerprints of any organism regardless of the complexity of the genome. The number of amplified fragments is controlled by the genome size, the cleavage frequency of the enzymes, and the number and nature of selective bases attached to the PCR primers. In order to optimize conditions for AFLP fingerprinting of *P. infestans* we varied (i) the restriction enzymes

(four base pair cutters) for digesting genomic DNA, (ii) the number of selective bases (2 or 3), and (iii) the PCR cycling profiles in the preamplification step and amplification step. The average size and the size distribution of fragments generated by digesting *P. infestans* DNA with *MseI*, *RsaI*, *Sau3AI*, or *TaqI* varies. The high GC content, which is presumed to be a general property of *Phytophthora* DNA, gave rise to a relatively large average size of *MseI* fragments and much smaller *RsaI*, *Sau3AI*, and *TaqI* fragments. Despite the large sized *MseI* fragments the best enzyme combination for AFLP fingerprinting with the lowest background appeared to be *EcoRI/MseI*. The best resolution for scoring was obtained with 4% acrylamide in the separating gel. For the extensions of the primers E+3/M+3, E+2/M+3, and E+2/M+2 were tested. The best results were obtained with E+2/M+2. Variation in the PCR profile included the annealing temperature and the number of cycles. A stepwise lowering of the annealing temperature in the preamplification step (a touchdown profile) resulted in the best patterns. The protocol for AFLP fingerprinting of *P. infestans* is described in detail under Materials and Methods. With this protocol the number of amplified fragments that is obtained in one assay is on average 87 and the sizes range from 70 to 1000 bp. In Figs. 1 and 2 sections of autoradiographs with AFLP fingerprints of *P. infestans* are shown.

### AFLP Analysis of the Parental Lines 80029 and 88133 of Cross 71

Prior to segregation analysis of AFLP markers in the F1 progeny of *P. infestans* cross 71, suitable primer combina-

**Selective restriction fragment amplification.** For the selective amplification of a limited number of DNA restriction fragments the secondary template DNA was amplified with primers containing two selective 3' nucleotides (E+2 and M+2 primers). Eleven primer combinations were used. In the E+2 primers the core sequence E was 5'-AGACTGCGTACCAATTC. In the M+2 primers the core sequence M was 5'-GATGAGTCCTGAGTAA. The following primer combinations were used: E+AA/M+AA, E+AA/M+AC, E+AA/M+AT, E+AA/M+CA, E+AA/M+CT, E+AC/M+CA, E+AC/M+CC, E+AC/M+CT, E+AG/M+AG, E+AG/M+CA, and E+AT/M+AG. The E+2 primers were labeled by phosphorylating the 5' end with [ $\gamma$ - $^{32}\text{P}$ ]ATP. Five microliters of the diluted secondary template DNA was mixed with 2.5 ng  $^{32}\text{P}$ -labeled E+2 primer, 30 ng unlabeled M+2 primer, 0.2 mM dNTPs and 0.7 U *Taq* DNA polymerase in 10  $\mu\text{l}$  10 mM Tris, pH 8.3, 1.5 mM  $\text{MgCl}_2$ , and 50 mM KCl. For the selective amplification the following PCR profile was used: the first cycle with 30 s at 94°C, 30 s at 65°C, and 1 min at 72°C was followed by 13 cycles with a stepwise lowering of the annealing temperature by 0.7°C in each cycle and 10 cycles with an annealing temperature of 56°C.

**Separation of labeled fragments and autoradiography.** After the second PCR, liquid was removed from the samples by evaporation. The DNA was dissolved in 10  $\mu\text{l}$  formamide dye (50% formamide, 10 mM EDTA with bromophenol blue and xylene cyanol as tracking dyes), denatured by heating for 3 min at 90°C, and quickly cooled on ice. Two microliters was loaded on 4% denaturing polyacrylamide gels (acrylamide:bisacrylamide ratio 20:1; gel buffer is 0.5 $\times$  TBE buffer, 7.5 M urea; 1 $\times$  TBE is 100 mM Tris, 100 mM boric acid, 2 mM EDTA, pH 8.0).  $^{32}\text{P}$ -labelled *Pst*I-digested  $\lambda$  DNA was loaded as size marker. The gels were run on a SequiGen 38  $\times$  50 cm gel apparatus (Bio-Rad Laboratories Inc., Richmond, VA) with 1 $\times$  TBE as electrophoresis buffer in the cathode buffer compartment and 1 $\times$  TBE, 0.5 M NaAc as electrophoresis buffer in the anode buffer compartment. Electrophoresis was performed at constant power, 110 W, for 2 h. The gels were dried on Whatmann 3MM paper and exposed to X-ray films (Kodak X-omat AR) for 1–5 days at room temperature.

**Nomenclature of AFLP markers.** AFLP markers were designated by the primer combination that was used to amplify the fragment and their mobility in the gel relative to DNA markers of known length. Since the mobility depends on length as well as nucleotide content the numbers do not represent the precise sizes of the

fragments but approximate sizes in nucleotides. The prefix A, B, or H indicates the origin of the marker as explained below.

## Data Analysis and Map Construction

AFLP fingerprints were scored visually. Two different types of data files were made. In one type the presence or absence of polymorphic DNA fragments was given in binary characters (1 or 0). These were used as input files for genetic cluster analysis using the software program NTSYS-pc (Rohlf, 1993). Genetic cluster analysis was performed to analyze genetic differences between single zoospore isolates and also to identify genetically identical individuals in the F1 progeny. F1 progeny with identical genotypes might be descendants from the same oospore and should therefore be considered as one F1 individual. Similarity was calculated using the Dice coefficient and for cluster analysis the UPGMA algorithm was used (Rohlf, 1993).

The other type of data files contained information about fragments that acted as segregating markers in the F1 progeny of cross 71. Table 1 shows how in a diploid, noninbred organism AFLP bands from parents of different genetic constitution can be inherited by the progeny. Fragments segregating according to genetic models 1, 3, 7, and 9 are not informative for mapping since all progeny are identical. The bands shown in the genetic models 2 and 4 can be considered as markers because they segregate in a 1:1 fashion. However, these markers can only be scored on the basis of intensity of the bands on the autoradiographs. We were restricted to visual scoring and since intensity scoring is time consuming and less reliable we excluded the type 2 and 4 markers. Type 6 and 8 AFLP markers (as shown in Table 1) were scored as A or B when absent (*aa*) and as H when heterozygous present (*Aa*). Type 5 AFLP markers were scored as H when heterozygous (*Aa*), as A when homozygous present (*AA*), as B when homozygous absent (*aa*), and as D when intensity differences did not allow discrimination between H (*Aa*) and A (*AA*).

In a similar way segregating RFLP markers were scored. These data were obtained from Southern blot hybridizations with two *P. infestans* DNA fingerprint probes, RG-57 (Goodwin *et al.*, 1992) and pPi122 (F. Govers, unpublished results). RFLP DNA fingerprinting was performed as described by Drenth and Govers (1993). The genotypes for mating type were designated *Aa* for A1 and *aa* for A2 (Shaw, 1991) and thus mating type was considered to be a type 6 marker. Marker types 5, 6, and 8 (Table 1) are indicated by the prefixes H, A, and B, respectively. H is a marker present in both parents whereas A is an A1 derived marker and B an A2 derived marker.

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## CHAPTER 3

### MAPPING OF AVIRULENCE GENES IN *PHYTOPHTHORA INFESTANS* WITH AMPLIFIED FRAGMENT LENGTH POLYMORPHISM MARKERS SELECTED BY BULKED SEGREGANT ANALYSIS

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# Mapping of Avirulence Genes in *Phytophthora infestans* With Amplified Fragment Length Polymorphism Markers Selected by Bulk Segregant Analysis

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## ABSTRACT

In this study we investigated the genetic control of avirulence in the diploid oomycete pathogen *Phytophthora infestans*, the causal agent of late blight on potato. The dominant avirulence (*Avr*) genes matched six race-specific resistance genes introgressed in potato from a wild *Solanum* species. AFLP markers linked to *Avr* genes were selected by bulked segregant analysis and used to construct two high-density linkage maps, one containing *Avr4* (located on linkage group A2-a) and the other containing a cluster of three tightly linked genes, *Avr3*, *Avr10*, and *Avr11* (located on linkage group VIII). Bulk segregant analysis also resulted in a marker linked to *Avr1* and this allowed positioning of *Avr1* on linkage group IV. No bulk segregant analysis was performed for *Avr2*, but linkage to a set of random markers placed *Avr2* on linkage group VI. Of the six *Avr* genes, five were located on the most distal part of the linkage group, possibly close to the telomere. The high-density mapping was initiated to facilitate future positional cloning of *P. infestans Avr* genes.

**R**ESISTANCE of plants to pathogens often depends on the activation of defense responses after pathogen attack. A key factor in this type of resistance is the perception of the pathogen by the host, which triggers the appropriate defense responses. When defense responses completely block pathogen development, the interaction between pathogen and plant is called incompatible. Genes from the pathogen that mediate recognition and activation of host defense responses leading to incompatible interactions are called avirulence genes. Incompatible interactions are usually associated with a hypersensitive response in the host and a high degree of specificity between the pathogen genotype and the host genotype. This high specificity is also known as race-specific resistance of the host or race-specific virulence of the pathogen and had been observed by Flor in the early 1940s in the flax-flax rust pathosystem (FLOR 1942; ELLIS *et al.* 1997). In the last decade Flor's gene-for-gene model, which explains the high specificity, gained support as a general mechanism governing plant-pathogen interactions. Single dominant avirulence genes were identified and cloned from a wide

range of pathogens (reviewed by BONAS and VAN DEN ACKERVEKEN 1999; LAUGÉ and DE WIT 1998), and dominant matching resistance genes were isolated from various plant species (reviewed by ELLIS and JONES 1998).

The subject of our studies is *Phytophthora infestans*, the causal agent of potato late blight and one of the economically most important pathogens of potato worldwide. *P. infestans* and potato interact according to the gene-for-gene model. Eleven major resistance genes (R-genes) introgressed from *Solanum demissum* provide strong resistance against specific races of the pathogen (BLACK 1954; EIDE *et al.* 1959; MALCOLMSON and BLACK 1966; MALCOLMSON 1969). These 11 R-genes suggest the presence of 11 corresponding virulence or avirulence factors in *P. infestans*, and genetic analyses on both host and pathogen have been performed to confirm the gene-for-gene model in this pathosystem.

In potato, the position on the genome of 5 out of 11 R-genes was determined by linkage analyses (R1, MEKSEM *et al.* 1995; R3, R6, and R7, EL-KHARBOTLY *et al.* 1996; R2, LI *et al.* 1998), thereby demonstrating that a single locus in the host governs race-specific resistance. The inheritance of virulence and avirulence in *P. infestans* is less clearly defined. SPIELMAN *et al.* (1989, 1990) analyzed segregation ratios of virulent and avirulent phenotypes in three F<sub>1</sub> progeny from matings involving five Mexican isolates and in some F<sub>2</sub> progeny and backcrosses. Their results were in favor of single locus control for (a)virulence against R1 and R2, but suggested involvement of more than one locus for (a)virulence against R3 and R4. A more extensive study by AL-KHERB *et al.*

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(1995) involving seven isolates, and segregation analyses in 10 F<sub>1</sub> crosses and in sib matings and backcrosses, revealed single locus control for (a)virulence against 10 of the 11 R-genes, while (a)virulence against R10 seemed to depend on two loci. However, a drawback of the studies by SPIELMAN *et al.* (1989, 1990) and AL-KHERB *et al.* (1995) is the lack of molecular markers linked to virulent or avirulent phenotypes, which hampers drawing firm conclusions. For example, segregation ratios may be obscured by the fact that not all oospores of *P. infestans* are viable (PITTIS and SHATTOCK 1994; AL-KHERB *et al.* 1995). Also, loss of pathogenicity in F<sub>1</sub> progeny occurs frequently and that excludes part of the progeny from virulence tests. Furthermore, analysis of F<sub>1</sub> progeny of *P. infestans* with molecular markers revealed the occasional occurrence of trisomic individuals (CARTER *et al.* 1999; T. VAN DER LEE and F. GOVERS, unpublished results). Thus, predictions based on segregation ratios alone should be treated with caution.

Here we present studies on the inheritance of race-specific virulence in *P. infestans*. Progeny of a cross in which six avirulence (*Avr*) genes segregate were investigated (ALFONSO and GOVERS 1995). This F<sub>1</sub> progeny was previously used to construct a genetic linkage map and the parental lines were shown to behave genetically as diploid homokaryons (VAN DER LEE *et al.* 1997). By means of bulked segregant analysis (BSA; MICHELMORE *et al.* 1991) amplified fragment length polymorphism (AFLP) markers tightly linked to five *Avr* genes were found. Linkage analysis showed that avirulence is dominant. By integrating the markers in the genetic linkage map (VAN DER LEE *et al.* 1997), all six *Avr* genes could be positioned on the map, and high-density maps of two genomic regions containing *Avr* genes were constructed.

## MATERIALS AND METHODS

**Nomenclature of genes and phenotypes:** Anticipating that the six avirulence genes analyzed in this study would be dominant, the nomenclature used for these genes (or gene loci) is *Avr* (with a capital A) for the avirulent genotypes and *avr* for the virulent genotypes. This is followed by a number indicating the corresponding host resistance gene (*e.g.*, *Avr1*). Consequently, the phenotypes are indicated by AVR and avr; *e.g.*, a strain with the AVR1 phenotype is avirulent on plants carrying the R1 resistance gene whereas an avr1 strain is virulent on R1 plants.

*Avr3*, *Avr10*, and *Avr11* were postulated to match the corresponding resistance genes R3, R10, and R11. Since there is no evidence that these three avirulence genes represent a single gene, they are treated as independent genes, and the locus in which the avirulence genes cluster is called *Avr3-Avr10-Avr11*.

***P. infestans* mapping population:** The mapping population consisted of 76 F<sub>1</sub> progeny from cross 71, a cross between two Dutch *P. infestans* isolates, 80029 (race 2.4.7; A1 mating type) and 88133 (race 1.3.7.10.11; A2 mating type). The progeny were derived from oospores generated in infected leaves (*in vivo*) and were recovered from sporulating lesions formed on leaves that were floated on water containing soil with oospores.

The mapping population was previously characterized by DRENTH *et al.* (1995) and VAN DER LEE *et al.* (1997). Sixty-eight progeny of this cross were included in the virulence assays.

**Virulence assays:** *P. infestans* isolates, stored as spores or mycelium plugs in 15% DMSO in liquid nitrogen, were transferred to 9-cm petri dishes containing rye agar medium supplemented with 2% sucrose (CATEN and JINKS 1968). When plates were fully covered with mycelium (after 1–2 weeks) small plugs were transferred to fresh medium. To isolate zoospores, sporulating cultures were flooded with 10 ml of demineralized water and incubated at 4° for 3 hr to allow formation and release of zoospores. The zoospore suspension was collected and kept on ice. The zoospore concentration was counted and the appropriate number was used for inoculation of potato leaves.

Potato lines of the differential set were obtained from Plant Research International and from the Laboratory of Plant Breeding of Wageningen University. We used the following lines (R-gene in parentheses): CEBECO43154-5 (R1), CEBECO44158-5 (R2), CEBECO4642-1 (R3), CEBECO4431-5 (R4), Black2182ef(7) (R7), Black3618ad(1) (R10), Black5008ab(6) (R11), CEBECO4739-58 (R1R3), and CEBECO5073-1 (R2R3). In addition we used the R-gene containing potato cultivars Ehud (R1), Saturna (R1), and Astarte (R1R3), and the cultivar Bintje, which has no R-genes (r0). Sterile plants were grown on Murashige and Skoog (MS) medium amended with vitamins and 2% sucrose at 20° and 16 hr light/8 hr dark. Top cuttings of plants were transferred to new medium and grown for 2 weeks under the regime mentioned above after which the plants were transferred to sterile soil in a climate chamber. In the first 2–3 days after transfer to soil, plants were covered with plastic. Plants were transferred to 5-liter pots 1–2 weeks after transfer to soil. Leaves were used for inoculation with *P. infestans* 8–10 weeks after transfer to soil.

Full-grown leaves were cut from the fourth to the seventh node and stuck in water-saturated flower foam. Leaves were transferred to trays with water-soaked filter paper at the bottom and transparent plastic lids to ensure high humidity in the tray. The lower side of the leaf was drop inoculated (~10 µl) with 10<sup>3</sup> or 2 × 10<sup>3</sup> zoospores. Per *P. infestans* strain and per R-gene differential at least eight inoculation spots with 10<sup>3</sup> and eight inoculation spots with 2 · 10<sup>3</sup> zoospores were analyzed in every test. Leaves were incubated for 4 days (16 hr at 18°/light and 8 hr at 15°/dark) before responses were scored. As explained in the results, five different classes of responses were distinguished. When different classes were observed at different inoculation sites the number in each class was counted.

**DNA isolation and AFLP DNA fingerprinting:** DNA isolation from *P. infestans* was performed as described previously by DRENTH and GOVERS (1993) with some minor modifications as described in VAN DER LEE *et al.* (1997). AFLP DNA fingerprinting was performed essentially as described by Vos *et al.* (1995) using the restriction enzyme combination *EcoRI*/*MseI* with two selective bases on each side, which was shown to generate highly informative fingerprints for *P. infestans* (VAN DER LEE *et al.* 1997). The nomenclature of the AFLP markers is as follows. The first letter, A, B, or H, indicates the origin of the marker (A for the A1 parent, B for the A2 parent, and H for fragments present in both parents). This is followed by E + XX/M + XX in which E and M refer to *EcoRI* and *MseI*, respectively, and XX to the extensions of the selective bases used. The numbers at the end (preceded by s) refer to the approximate size of the fragment in base pairs. For example, the marker AE + AG/M + ATs400 is present only in the A1 parent; the fragment was generated using an *EcoRI*/*MseI* restriction digest, amplified with a primer with an AG extension on the *EcoRI* site and a primer with an AT extension on the *MseI* site, and has an estimated size of 400 bp.

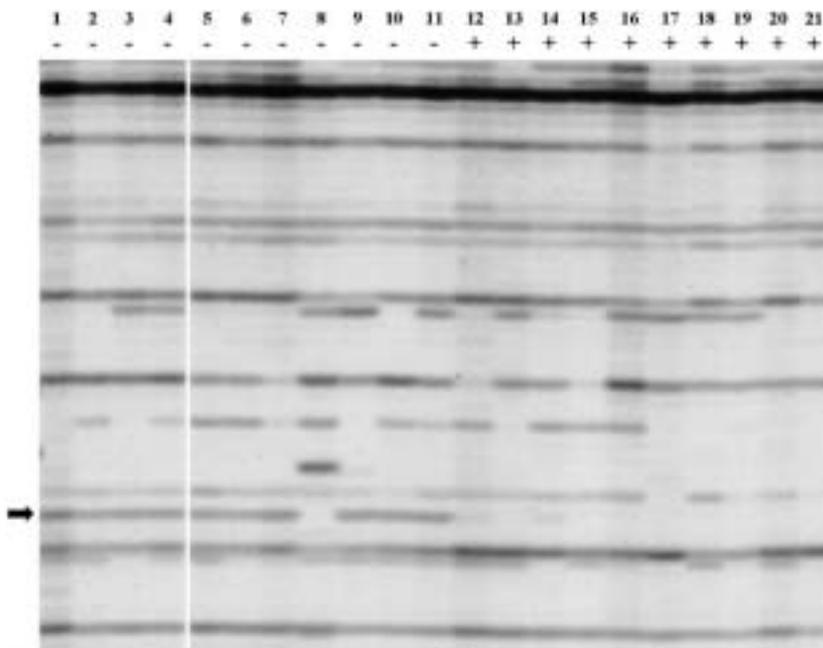


FIGURE 1.—Segregation of an *Avr4* linked marker in cross 71. Section of an autoradiograph showing AFLP DNA fingerprints generated using the primer combination E + AC/M + TT. The parental line 88133 (lane 6) and progeny T15-1 (lane 1), T30-2 (lane 2), T30-4 (lane 3), T30-5 (lane 5), RE11-8 (lane 8), RE11-12 (lane 9), and RE11-16 (lane 10) are avirulent on R4 plants. The parental line 80029 (lane 18) and progeny T15-2 (lane 12), T15-4 (lane 13), T30-7 (lane 14), RT15-3 (lane 16), RE11-9 (lane 17), T35-3 (lane 20), and RE11-14 (lane 21) are virulent on R4 plants. Lanes 4, 7, and 11, and lanes 15 and 19 are fingerprints from pooled avirulent and virulent progeny, respectively. The arrow on the left indicates an AFLP marker for *Avr4* (BE + AC/M + TTs165). Virulence and avirulence phenotypes are indicated by + and -, respectively. The absence of this marker in progeny E11-8 (lane 8) suggests a recombination between this marker and *Avr4*.

**BSA:** Two rounds of BSA were performed essentially according to the procedure described by MICHELMORE *et al.* (1991). One round was aimed at generating markers located in the *Avr3-Avr10-Avr11* region and the other at generating markers linked to *Avr1* and *Avr4*. In the screening for markers linked to *Avr3*, *Avr10*, and *Avr11* four pools of progeny with two different combinations of phenotypes were used. Pools 1 and 4 represented the phenotype *avr3;avr10;avr11* and pools 2 and 3 the phenotype *AVR3;AVR10;AVR11*. The pools contained the following F<sub>1</sub> progeny: pool 1, D12-12, T15-1, T30-2, T35-3, and T35-4; pool 2, T15-2, T15-5, T15-7, and T15-9; pool 3, D12-9, D12-17, E12-3, E12-7, and T20-2; pool 4, D12-18, D12-21, D12-23, D12-25, E12-2, E12-15, and E12-22.

In the screening for markers linked to *Avr1* and *Avr4* six pools were used with the following combinations of phenotypes and F<sub>1</sub> progeny: pool 1, *AVR1;AVR4* (F<sub>1</sub> progeny T15-1, T30-2, and T30-4); pool 2, *avr1;AVR4* (F<sub>1</sub> progeny T20-2 and T80-3 and parent 88133); pool 3, *AVR1;AVR4* (F<sub>1</sub> progeny RE11-8, RE11-12, and RE11-16); pool 4, *avr1;avr4* (F<sub>1</sub> progeny T15-2, T15-4, and T30-7); pool 5, *AVR1;avr4* (F<sub>1</sub> progeny RE11-09 and RT15-3 and parent 80029); pool 6, *avr1;avr4* (F<sub>1</sub> progeny, RE11-14, RE11-15, and T35-03). Before starting the BSA all individuals were tested for contribution to the pools. AFLP fingerprinting was performed on pools and on individuals, and the contribution of each individual to the pool was balanced by adding more or less template. In both rounds of BSA, all 256 possible *EcoRI* + 2/*MseI* + 2 primer extensions 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 individual progeny of cross 71. An example is shown in Figure 1.

**Mapping of avirulence genes:** Linkage analysis and mapping were performed using the mapping software JoinMap 2.0 (STAM 1993). Maps were constructed using an LOD linkage threshold value of 4.5. Mapping of dense clusters of markers is not always straightforward. Small inconsistencies in the data set result in strong friction as indicated by the  $\chi^2$  value. To reduce this friction JoinMap occasionally positioned markers outside this dense region to a region containing less markers. However, if the LOD values clearly indicated that the marker

was located in the dense region, the  $\chi^2$  frictions were overruled and the markers were manually positioned in the dense regions.

## RESULTS

**Segregation of race-specific virulence in cross 71:** F<sub>1</sub> progeny of a cross between two Dutch field isolates (cross 71: A1 mating-type parent 80029, race 2.4.7, and A2 mating-type parent 88133, race 1.3.7.10.11) were tested for virulence on a differential set of potato lines carrying the major R-genes R1, R2, R3, R4, R10, and R11, respectively. In the progeny virulence against these six R-genes segregates (ALFONSO and GOVERS 1995).

The scoring for virulence or avirulence is hampered by the fact that the differential set of potato lines is not genetically uniform and that the infection severity is not identical on all potato lines. Also, the *P. infestans* progeny appeared to be variable in aggressiveness on potato, and therefore in every virulence assay the aggressiveness on a potato cultivar without R-genes (r0) was analyzed. If the progeny was not able to infect r0 plants it was not included in the segregation analysis. Four days after inoculation, five different macroscopic responses were distinguished: (A) no symptoms, (B) dark localized necrosis, (C) spreading lesions without sporulation, (D) spreading lesions with some sporangiospores, and (E) spreading lesions with massive sporulation. On the R1 and R3 potato lines the interaction was rated compatible if the responses were of classes D and E. Class A or B responses were considered to be incompatible interactions and class C responses were rated unknown. These results were confirmed by virulence assays on the cultivars Ehud (R1), Saturna (R1), and Astarte (R1R3). The same rating was used for the virulence on R10 and

**TABLE 1**  
**Segregation of race-specific virulence in the progeny of *P. infestans* cross 71**

R-gene	Phenotypes <sup>a</sup>						$\chi^2$ <sup>c</sup>	<i>P</i> <sup>c</sup>
	Parents		Progeny		<i>N</i> <sup>b</sup>			
	80029	88133	Observed +:–	Expected +:–				
1	–	+	24:33	1:1	57 (63)	1.42	0.23	
2	+	–	21:39	1:1	60 (62)	5.40	0.02	
3	–	+	30:21	1:1	51 (62)	1.00	0.32	
4	+	–	30:23	1:1	53 (63)	0.92	0.34	
10	–	+	24:27	1:1	51 (62)	0.18	0.67	
11	–	+	25:25	1:1	50 (62)	0.00	1.00	

<sup>a</sup> +, virulent; –, avirulent.

<sup>b</sup> Number categorized; in parentheses, number tested.

<sup>c</sup> The  $\chi^2$  and the corresponding *P* value were calculated to test the probability that the data fit an expected ratio of 1:1 for segregation of a single gene conferring avirulence.

R11 lines but here symptoms were less severe when compared to those on R1 and R3 lines. Symptoms on the R2 potato line were also less severe; class E responses were not found; class C and D responses were rated compatible while class A and B responses were rated incompatible. Symptoms on R4 potato lines were more severe. Class E responses were rated compatible; class D responses were rated unknown; and class A, B, and C responses were rated incompatible. The scoring of virulence on the differential set was reproducible and in line with what was reported previously (ALFONSO and GOVERS 1995).

As shown in Table 1, segregation ratios for virulence/avirulence on potato lines carrying the R1, R3, R4, R10, or R11 resistance gene did not differ significantly from 1:1. However, a significant deviation from the expected 1:1 ratio toward avirulence was found on R2 plants; here the ratio was close to 1:2.

**Fine mapping of *Avr3*, *Avr10*, and *Avr11* using bulked segregant analysis:** Initial analysis of the progeny showed genetic linkage of virulence on potato lines carrying R3, R10, or R11. For BSA we constructed two pools of AFLP templates from progeny avirulent on R3, R10, and R11 plants and two pools of AFLP templates from progeny virulent on those plants. The number of progeny in the pools varied from four to seven. The pooled templates were fingerprinted by AFLP using all 256 combinations of *EcoRI* + 2/*MseI* + 2 primer extensions. Over 25,000 AFLP fragments were analyzed and, on the basis of previous experiments (VAN DER LEE *et al.* 1997), it is estimated that these include over 1250 markers segregating in the cross 71 progeny and originating from the A1 parent (Aa × aa). BSA yielded 20 fragments that were specific for the avirulent pools. These candidate markers were tested on the individuals of the bulks and on other progeny of cross 71. Fifteen of the 20 markers (75%) showed linkage to *Avr3*, *Avr10*, and *Avr11* and most were within 10 cM distance (Table 2). The direct distances

between the markers and the avirulence loci are ~8 cM with LOD values ranging from 5 to 6. For this region no additional markers were found in a random set of 240 markers. All linked markers and *Avr3*, *Avr10*, and *Avr11* map on the distal part of linkage group VIII (Figure 2C). The calculated map distances between markers in the region and *Avr3*, *Avr10*, and *Avr11* were smaller than the direct distances. This difference between the direct distance and the map distance caused some friction in the map, but the LOD values and the direct distances clearly positioned *Avr3*, *Avr10*, and *Avr11* in the region.

**Fine mapping of *Avr1* and *Avr4* using bulked segregant analysis:** *Avr1* and *Avr4* segregate as independent loci in cross 71. Nevertheless, a BSA was set up that allowed identification of linked markers for both loci simultaneously. Six pools with AFLP templates from progeny with the following phenotypes were composed: AVR1;AVR4 (two pools with three individuals), AVR1;avr4 (one pool with three individuals), avr1;AVR4 (one pool with three individuals), and avr1;avr4 (two pools with three individuals). In this way markers linked to *Avr1* could be distinguished from markers linked to *Avr4*. Over 30,000 AFLP fragments were analyzed, resulting in 23 candidate markers for *Avr1* and 16 candidate markers for *Avr4* (Table 2). Only one of the *Avr1* markers, AE + CG/M + TGS317, appeared to be linked when tested on the individual progeny of the bulks and on additional progeny of cross 71. Linkage analysis with all markers currently identified in the cross 71 mapping population showed linkage of this marker with a marker distal on linkage group IV (Figure 2A). The direct distance between the marker and *Avr1* is 8 cM and the corresponding LOD value is 10.4.

Initially, 16 candidate markers were identified for *Avr4*, of which 7 appeared to be linked. Segregation of 1 linked marker is shown as an example (Figure 1). One additional marker was identified in a set of 240

TABLE 2

Number of candidate markers selected by BSA and number of markers linked to *Avr* genes

Avirulence gene	No. of AFLP fragments analyzed	Estimated no. of informative markers <sup>a</sup>	Candidate markers after BSA	Markers linked (<10 cM)
<i>Avr1</i>	>30,000	1,500	23	1
<i>Avr2</i>	>4,000	240 <sup>b</sup>	NR <sup>c</sup>	7
<i>Avr3-Avr10-Avr11</i>	>25,000	1,250	20	15
<i>Avr4</i>	>30,000	1,500	16	7 + 1 <sup>d</sup>

<sup>a</sup> Informative markers are markers that segregate in the cross 71 progeny.

<sup>b</sup> For *Avr2* no BSA was performed; consequently, the number of informative fragments is not an estimate but the real number.

<sup>c</sup> Not relevant.

<sup>d</sup> Seven from BSA and one from the set of random markers.

random markers. The *Avr4* locus could be mapped distally on A2-a, a linkage group containing only markers from the A2 parent (Figure 2D). The direct distance between *Avr4* and the closest marker is 2 cM with a LOD value of 12.4.

**Mapping of *Avr2*:** The *Avr2* gene was mapped with random markers. These markers were derived partly from the set generated to construct the first genetic map (VAN DER LEE *et al.* 1997) and partly from markers that were obtained when primer combinations used to test the candidate markers for the other five *Avr* genes were analyzed on all progeny of cross 71 individually. In the latter fingerprints, on average, 15 segregating markers could be scored in addition to the candidate marker. In total, 470 of these markers, of which 240 were derived from the avirulent 88133 parental line, were tested for linkage to virulence on the R2 potato line

(Table 2). Seven markers derived from the A2 parent appeared tightly linked to *Avr2* and, as a result, *Avr2* could be positioned on linkage group VI (Figure 2B).

**Avirulence is a dominant trait:** Analysis of the diploid F<sub>1</sub> progeny of an outbreeding cross allows discrimination between dominant and recessive traits if phenotypes can be mapped and if the phenotypes of the parents are known. Linkage can only be found between markers or traits from the same parent. In the progeny of cross 71 we found clear linkage between avirulence on R1, R3, R10, or R11 potato lines and markers from parent 80029. This indicates that the gamete of this parent determines whether the progeny will be virulent or avirulent on R1 or on R3, R10, and R11 potato lines. Since parent 80029 itself is avirulent on these lines, avirulence is dominant. Similarly, from the linkage of avirulence on R2 or R4 potato lines with markers from

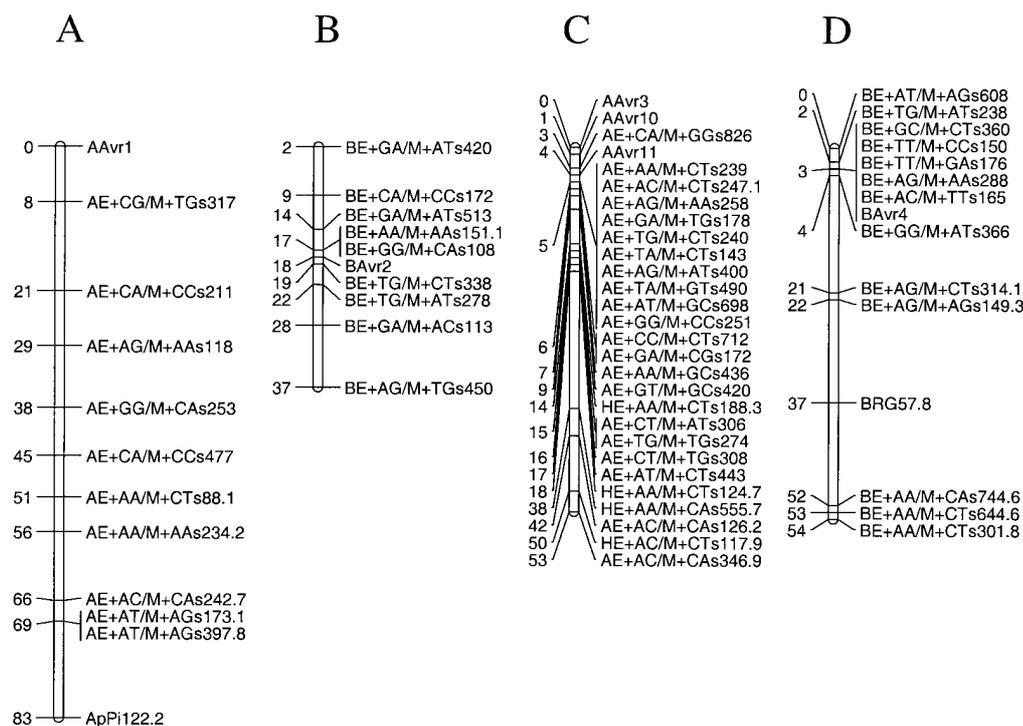


FIGURE 2.—Genetic maps of linkage groups containing avirulence genes. Maps were constructed using JoinMap 2.0 (STAM 1993) with a link LOD threshold of 4.5 and a map LOD threshold of 0.01. Markers are indicated on the right, cumulative map distances (in centimorgans) on the left. (A) Linkage group IV containing *Avr1*. (B) Linkage group VI containing *Avr2*. (C) Linkage group VIII containing *Avr3*, *Avr10*, and *Avr11*. (D) Linkage group A2-a containing *Avr4*.

parent 88133, we conclude that the gamete of 88133 determines the phenotypes on R2 and R4. Parent 88133 is avirulent on potato plants carrying the R2 and R4 gene, and consequently *Avr2* and *Avr4* are dominant.

## DISCUSSION

*P. infestans* populations are notorious for the appearance of new virulent races that are able to overcome monogenic resistances introduced in potato. To gain insight into the genetic mechanisms underlying race specificity, the inheritance of avirulence genes in *P. infestans* was studied. Here we report the mapping of six race-specific avirulence genes in the sexual progeny of two Dutch field isolates. AFLP DNA fingerprinting was used in combination with BSA to identify markers tightly linked to these avirulence genes, and the relevant chromosomal regions were saturated with DNA markers, a prerequisite for positional cloning of avirulence genes.

For each of the six tested R-genes, a single corresponding avirulence locus was identified that could be positioned on the genetic linkage map. Our analyses, however, indicate that, in addition to these major factors, minor factors influence the responses on the potato lines that comprise the differential set. The genetic background of the lines is not uniform and different levels of "basal" resistance were observed. The potato lines carrying resistance genes R1, R3, or R4 were easily infected by virulent *P. infestans* isolates. Under high disease pressure, for example, resistance in the R4 differential was occasionally lost. On the other hand, the lines with R10, R11, and certainly R2 appeared to be less susceptible. All the progeny in which no correlation was found between the closest linked markers and *Avr2* are avirulent, indicating that this differential may contain other resistance genes. This is supported by the aberrant segregation ratio showing a significantly higher number of avirulent progeny than expected (Table 1). To reduce the variability caused by environmental factors and by genetic differences in the differential set other than the R-genes, the plants were grown in controlled conditions in climate chambers, high concentrations of zoospores were used, and scoring was done at early time points in disease development. In cases where the response was doubtful, it was rated as unknown, and this contributed to the reliability of the linkage analysis.

Since no avirulence genes of *P. infestans* were mapped previously, the pools for the BSA experiments were carefully designed. Approximately 20 progeny were divided over at least four pools. This allowed us to screen in different genetic windows and enabled us to neutralize an incorrectly scored individual, once identified, in one of the pools. Initial analysis showed that the use of at least four relatively small pools allowed reliable identification of markers with two recombinants in the pools, providing a genetic window of 10 cM (2 out of 20). Because two or three phenotypes were screened simulta-

neously, the number of progeny in the pools was limited to minimize incorrect scorings of individuals in the pools for one of the traits. It was estimated previously that the genome size of *P. infestans* in cross 71 is ~1200 cM (VAN DER LEE *et al.* 1997). With a genetic window of 10 cM and the screening of 30,000 AFLP markers, of which 5% would be a segregating marker for either the 80029 or 88133 parent, ~10 markers were expected for a target gene at the end of a linkage group and 20 linked markers for a gene in the middle of a linkage group. Screening for markers linked to *Avr3*, *Avr10*, and *Avr11* yielded more markers than expected (*i.e.*, 15), whereas the BSA for *Avr1* linked markers resulted in a remarkably low number of markers, *i.e.*, only 1. For *Avr4*, 7 linked markers were found, which is a bit less than expected. For *Avr2*, no BSA was carried out. Yet, by analyzing linkage of *Avr2* with random markers we identified more markers linked to *Avr2* than to *Avr1* (7 *vs.* 1).

The BSA for *Avr3*, *Avr10*, and *Avr11*, as well as for *Avr4*, was efficient; 75% of the candidate markers were indeed linked to the *Avr3-Avr10-Avr11* cluster, and nearly 45% of the candidate markers for *Avr4* were truly linked. This was different in the screening for *Avr1*. A large number of candidate markers were identified, but only one appeared to be linked. It is not very likely that this is caused by the involvement of a second locus or by less reliable virulence data. Avirulence on R1 potato lines segregated in a 1:1 ratio, and R1 is the most reliable differential in the set. Moreover, the virulence data were confirmed using potato cultivars Ehud (R1), Saturna (R1), and Astarte (R1R3), and the fact that one tightly linked marker was identified contradicts incorrect scorings in the virulence assays. In the same BSA round we successfully selected a number of *Avr4* linked markers, eliminating the chance that technical problems are the cause. The reason for the relatively low number of markers in the *Avr1* region may be low polymorphism of the homologous chromosomes in the *Avr1* region in the 80029 parent or a high recombination frequency. Likewise, the relatively high number of markers linked to *Avr2* and the *Avr3-Avr10-Avr11* cluster might be explained by high rates of polymorphism or recombination suppression in these chromosomal regions. Even though marker densities differ in different regions of the cross 71 map constructed by linkage analysis (VAN DER LEE *et al.* 1997), the large differences observed in this study in the various regions surrounding avirulence genes are exceptional.

All 256 *EcoRI* + 2/*MseI* + 2 primer combinations were tested in the two rounds of BSA, one for *Avr1* and *Avr4* and the other one for *Avr3*, *Avr10*, and *Avr11*. If no markers were overlooked in the BSA, no additional markers would be identified in the random set of markers generated by the 50 primer combinations tested on all progeny. For *Avr1* and the *Avr3-Avr10-Avr11* cluster, this was indeed the case but for *Avr4* one additional

marker was found. This demonstrated that the BSAs were sufficiently systematic.

Our analyses clearly show that avirulence is a single dominant trait for all six segregating *Avr* genes tested in cross 71. Most fungal and bacterial *Avr* genes studied so far act in a dominant fashion (reviewed by LAUGÉ and DE WIT 1998) and indeed if avirulence genes encode proteins with elicitor function, dominance is to be expected. However, unlike the reverse genetics approach that is often used for cloning fungal avirulence genes (JOOSTEN *et al.* 1994), genetic analysis might also reveal genes that do not themselves encode elicitors but, instead, mediate the synthesis of elicitors such as transcriptional regulators, enzymes, or transporters. These factors may act in a dominant or recessive fashion. Therefore, the conclusion by SPIELMAN *et al.* (1989) that virulence in *P. infestans* on R4 plants is dominant does not necessarily contradict our findings. Since they studied segregation in other crosses, they may have analyzed another locus determining the AVR4 phenotype but, obviously, this locus does not segregate in cross 71.

High-density mapping confirmed the tight clustering of *Avr3*, *Avr10*, and *Avr11* that was noted at the phenotype level in the virulence assays. Linkage of *Avr* genes in *P. infestans* was reported before (AL-KHERB *et al.* 1995; CARTER *et al.* 1999) but the tight clustering of *Avr3*, *Avr10*, and *Avr11* was not observed, even though the three avirulence phenotypes segregated in the crosses examined by AL-KHERB *et al.* (1995). This not only raises the question of whether we have been analyzing the same or different factors as AL-KHERB *et al.* (1995), but also whether we are really dealing with three different independent genes at the same locus. Tight clustering of *Avr* genes in plant pathogens is not uncommon. Cosegregation of avirulence genes was reported for *Avr4* and *Avr6* as well as for *Avr1b* and *Avr1k* in *P. sojae* (WHISSON *et al.* 1995; GIJZEN *et al.* 1996). The rice blast fungus *Magnaporthe grisea* contains one cluster with three and another with two *Avr* genes (DIOH *et al.* 2000). Also, in the leaf blotch pathogen *Mycosphaerella graminicola*, avirulence phenotypes on genetically distinct cultivars of wheat appear to be linked at the genetic level (G. H. J. KEMA, personal communication). In none of these cases have the corresponding R-genes been cloned, so their primary structure is still unknown. It may well be that R-genes with different names or numbers represent the same R-gene in a different genetic background or are just slightly different but have the same *Avr* specificity. In both cases, a supposed *Avr* gene cluster might just be a single gene encoding an elicitor that is recognized by the highly homologous R-genes. The *Avr3-Avr10-Avr11* cluster can also represent a single regulatory gene encoding, *e.g.*, a positive regulator or modifier, and, as such, controlling other loci involved in avirulence against R3, R10, and R11. Presumably these loci are homozygous in the parental lines of cross 71 but heterozygous in some of the crosses used by AL-KHERB *et al.*

(1995). This is consistent with their explanation that the observed aberrant segregation ratios with regard to virulence on R10 potato plants may be caused by a dominantly acting gene. The involvement of regulatory loci in determining race-specific virulence has been postulated for other pathogens. In *Melampsora lini*, the causal agent of flax rust, two dominant inhibitor loci were found. One compromises resistance provided by the resistance alleles L1, L7, L8, L10, and M1 whereas the other locus inhibits resistance provided by M1 (JONES 1988; ELLIS *et al.* 1997). The gene involved might be a suppresser interfering with the race-specific resistance of the host, but it might also be a suppressor of avirulence genes. Alternatively, an *Avr* gene cluster may contain distinct genes that belong to a gene family and encode structurally related proteins. Such gene clusters do exist in *P. infestans*. One example is the *ipiB* gene family with three distinct members located on a 5-kb fragment (PIETERSE *et al.* 1994). Another example is the *elicitin* gene family that consists of two gene clusters (R. Y. H. JIANG and F. GOVERS, unpublished results) and whose members encode species-specific avirulence factors (KAMOUN *et al.* 1998).

Molecular cloning of the *Avr3-Avr10-Avr11* cluster and identification of the encoded protein(s) will reveal the true nature of the avirulence factors involved in R3, R10, and R11 resistance and may explain why these genes are clustered. Whatever the explanation is, stacking of R-genes to obtain broader resistance, but without knowledge of the genetic and molecular basis of avirulence, may not give the desired result. Complete loss of resistance may be caused by a single mutation if the supposed gene cluster appears to be just one gene or by a single deletion of the *Avr* gene cluster itself. Five out of six *Avr* genes in this study (*i.e.*, *Avr1*, *Avr3*, *Avr4*, *Avr10*, and *Avr11*) map on the most distal part of the linkage groups, probably close to the telomere. Taking into consideration that in eukaryotes, telomeric regions are among the most flexible regions in the genome, there may be a reasonable chance that avirulence is lost because of a deletion. In *M. grisea* a relatively large number of avirulence genes map near telomeres (SMITH and LEONG 1994; VALENT and CHUMLEY 1994; MANDEL *et al.* 1997; DIOH *et al.* 2000) and in one case, indeed a deletion causes a phenotypic change from avirulent to virulent (MANDEL *et al.* 1997). Mapping of telomeres might thus be instrumental in positioning avirulence genes. A telomeric repeat of *P. infestans* was cloned (PIPE and SHAW 1997), and it will be interesting to test its linkage to the *Avr* genes studied here and to other avirulence genes in *P. infestans*. Overall, identification of unstable genomic regions might contribute to the assessment of the flexibility of pathogens, particularly with regard to pathogenicity genes and avirulence genes (STRINGER 1996; FREITAS-JUNIOR *et al.* 2000), and therefore to the assessment of the durability of the corresponding resistance genes.

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## CHAPTER 4

# PHYSICAL MAPPING ACROSS AN AVIRULENCE LOCUS OF *PHYTOPHTHORA INFESTANS* USING A HIGHLY REPRESENTATIVE, LARGE-INSERT BACTERIAL ARTIFICIAL CHROMOSOME LIBRARY

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## Physical mapping across an avirulence locus of *Phytophthora infestans* using a highly representative, large-insert bacterial artificial chromosome library

**Abstract** The oomycete plant pathogen *Phytophthora infestans* is the causal agent of late blight, one of the most devastating diseases of potato worldwide. As part of efforts to clone avirulence (*Avr*) genes and pathogenicity factors from *P. infestans*, we have constructed a bacterial artificial chromosome (BAC) library from an isolate containing six *Avr* genes. The BAC library comprises clones with an average insert size of 98 kb and represents an estimated 10 genome equivalents. A three-dimensional pooling strategy was developed to screen the BAC library for amplified fragment length polymorphism (AFLP) markers, as this type of marker has been extensively used in construction of a *P. infestans* genetic map. Multiple positive clones were identified for each AFLP marker tested. The pools were used to construct a contig of 11 BAC clones in a region of the *P. infestans* genome containing a cluster of three avirulence genes. The BAC contig is predicted to encompass the *Avr11* locus but mapping of the BAC ends will be required to determine if the *Avr3* and *Avr10* loci are also present in the BAC contig. These results are an important step towards the positional cloning of avirulence genes from *P. infestans*, and the BAC library represents a valuable resource for large-

scale studies of oomycete genome organisation and gene content.

**Keywords** Plant pathogen · Genomics · Oomycete · Tomato

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### Introduction

*Phytophthora infestans* is the causal agent of late blight of potato and tomato, and is responsible for significant losses of these crops worldwide (Erwin and Ribeiro 1996). This is particularly the case for the cultivated potato, where the organism is regarded as a threat to global food security. *P. infestans* belongs to the oomycetes, a class of organisms that includes many important plant pathogens. The oomycetes exhibit a mycelial growth habit but are distinct from the true fungi (Hawksworth et al. 1995; Erwin and Ribeiro 1996). *P. infestans* exhibits a gene-for-gene interaction with potato, in which avirulence gene (*Avr*) products from the pathogen are recognised by the host plant expressing the cognate resistance gene (van der Lee et al. 2001). This leads to the hypersensitive response (HR) – localised programmed cell death – in the plant that inhibits further spread of the pathogen.

At the molecular level, *P. infestans* is becoming a model oomycete for the study of pathogenicity mechanisms and avirulence. Central to these studies are resources such as a molecular-genetic linkage map (van der Lee et al. 1997), an expressed sequence tag (EST) database (Kamoun et al. 1999), as well as techniques for the analysis of gene function, such as transformation (reviewed in Judelson 1997), gene silencing (van West et al. 1999), *in planta* reporter systems (Kamoun et al. 1998) and heterologous expression of genes from other *Phytophthora* species (Panabieres et al. 1998). DNA libraries, particularly large-insert genomic libraries, are a critical resource for structural genomics and, by definition, for positional cloning.

The system most amenable to the manipulation of large cloned DNA inserts is the *Escherichia coli* bacterial

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artificial chromosome (BAC; Shizuya et al. 1992). These cloning vectors, based on a single-copy F factor plasmid, are capable of stable maintenance of insert DNA of up to 300 kb in length. Insert sizes above 100 kb are preferred for positional cloning applications, since fewer clones are required for chromosome walking across, or chromosome landing within, linkage map intervals containing genes of interest. This is of particular importance if the genome concerned is large, or if the gene of interest lies in a genomic region where the frequency of recombination is low. The genomes of many fungal and oomycete plant pathogens are relatively small compared to those of other organisms in which positional cloning has been undertaken (Voglmayr and Greilhuber 1998). However, the genome size of *P. infestans* has been estimated to be 250 Mb (Tooley and Therrien 1987), which is relatively large for an oomycete plant pathogen, and larger than the genome of the model plant *Arabidopsis thaliana*.

When producing a BAC library that is to be used primarily for the positional cloning of multiple target genes, it is more efficient to use a heterozygous individual from a selected F<sub>1</sub> mapping population as a source of high-molecular-weight DNA. An F<sub>1</sub> from a mapping population that possesses all required markers and phenotypic traits can then be selected if all the target genes segregate in the population. Recently, using a segregating population, the positions of six dominant *Avr* genes have been located on the molecular genetic linkage map of *P. infestans*. All have tightly linked AFLP markers (van der Lee et al. 2001). Using one F<sub>1</sub> from this population, which contains all six *Avr* genes from the genetic cross described by van der Lee et al. (1997, 2001), we set out to (1) produce a BAC library representing a ten-fold coverage of the genome with an average insert size of 100 kb, and (2) construct a contig of BAC clones across one specific region of the *P. infestans* genome that contains a large number of AFLP markers and a cluster of *Avr* genes. This BAC library will be a valuable resource for positional cloning and for large-scale studies of oomycete genome organisation and gene content. Chromosome landing near an avirulence locus is an important step towards the cloning and characterisation of these genes, which are of crucial importance in the interaction of potato with *P. infestans*.

## Materials and methods

### *P. infestans* isolates

The F<sub>1</sub> individual T30-4, obtained from the genetic cross used to construct the linkage map of *P. infestans* (van der Lee et al. 1997), contains all six avirulence genes (*Avr1*, *Avr2*, *Avr3*, *Avr4*, *Avr10* and *Avr11*) segregating in the cross, and was used as a source of high-molecular-weight DNA for construction of the BAC library. The parental isolates 80029 (race 2.4.7; A1 mating type) and 88133 (race 1.3.7.10.11; A2 mating type) were used for verification of AFLP alleles present in the BAC library. Cultures were maintained on rye agar plates (Ribiero 1978) at 18°C. Sporangia and mycelial frag-

ments, scraped from the agar plate cultures, were used to inoculate pea broth for large-scale preparation of mycelium.

### Preparation of high-molecular-weight DNA from *P. infestans*

Three day old pea-broth cultures of T30-4 were filtered through Miracloth (Calbiochem) and the young mycelium protoplasted as described by Judelson et al. (1993). Typically, *P. infestans* mycelium was completely digested into protoplasts after 45 min at 25°C. Protoplasts were harvested, washed, embedded in agarose, and lysed as described by Judelson et al. (1993). Agarose plugs containing intact chromosomal DNA were then washed twice in 0.5 M EDTA pH 8.0 at 4°C, twice in 50 mM EDTA pH 8.0 at 4°C, and five times in TE buffer (10 mM TRIS-HCl, 1 mM EDTA) pH 8.0 at 4°C. Plugs were stored at 4°C in TE buffer prior to use.

### Partial restriction digestion of high-molecular-weight DNA

Optimal conditions for partial digestion of the high-molecular-weight DNA were determined by titrating the restriction endonuclease (*Hind*III or *Bam*HI) against the DNA. An agarose plug containing high-molecular-weight DNA was reduced to a fine slurry with a razor blade on a microscope slide. This slurry was resuspended in 1 ml of TE buffer and 4 µl of 25% Triton X-100, collected by brief low-speed centrifugation in a microcentrifuge, and excess TE buffer was removed. Aliquots of 50 µl were transferred to microcentrifuge tubes and equilibrated on ice for 1 h with the appropriate restriction endonuclease buffer, BSA (100 µg/ml), and spermidine (4 mM). Enzyme dilutions in 5 µl (1 U, 0.5 U, 0.25 U, 0.125 U, 0.06 U, and 0 U) were added to each tube and allowed to equilibrate on ice for 30 min. Restriction digests were then carried out at 37°C for 30 min, stopped by addition of EDTA to 50 mM, and kept on ice until loaded on a CHEF gel. Restriction fragments were separated on a 1% agarose gel in 1×TAE buffer, using a CHEF Mapper (Bio Rad) with a constant 20-s pulse time, an 18-h run time at 6 V/cm, a 120° included angle, and a constant temperature of 13°C. After staining in ethidium bromide, the optimal digest conditions were identified as those yielding the greatest amount of DNA in the range of 100–250 kb. For large-scale digestion, the conditions determined for the partial digestion were scaled up, except that the reaction volumes were kept at 70 µl, so as to reproduce as closely as possible the optimal digestion conditions. Restriction fragments were separated as described above. DNA was recovered from the gel by electroelution (5 V/cm, 4°C, 1×TAE, 3 h) from gel fragments punched out of the size range 130–250 kb using Quik-Pik electroelution capsules (Stratagene). Alternatively, DNA was electrophoresed on a second CHEF gel as follows. The section of the gel containing the partially digested DNA between 120–250 kb was excised and transferred to the top of a new 1% agarose CHEF gel, but in the reverse orientation such that the smallest fragments were located at the top of the gel. Electrophoresis was then performed using the same CHEF conditions as previously. This had the effect of concentrating partially digested DNA into a single band which was easier to recover, at a higher concentration, using electroelution. TAE buffer was removed by drop dialysis on 0.2 µm membranes (Millipore) against TE buffer at 4°C for at least 3 h. Concentration of the DNA samples, if required, was also performed by drop dialysis against 0.5×TE buffer containing 30% polyethylene glycol 8000 (Sigma).

### Preparation of the pBeloBAC11 vector

The BAC vector pBeloBAC11 (Kim et al. 1996) was prepared on a large scale from a 5 l LB broth culture containing 12.5 µg/ml chloramphenicol, which was grown for 24 h before harvesting. DNA was isolated using the Qiagen Giga Prep procedure for very low-copy number plasmids. Contaminating *E. coli* genomic DNA was degraded using Plasmid Safe exonuclease (Epicentre Technologies). pBeloBAC11 was digested with a ten-fold excess of either

*Hind*III or *Bam*HI. Dephosphorylation with shrimp alkaline phosphatase (Roche) was carried out at the same time as the restriction digest.

#### Ligation of *P. infestans* DNA into pBeloBAC11

For optimal ligation of partially digested DNA into BAC vectors, the conditions determined by Osoegawa et al. (1998) were used: a vector:insert ratio of 10:1, and final concentrations of 0.5 and 1 ng/ $\mu$ l of vector and insert DNA, respectively. Digested, dephosphorylated vector (10 ng) was tested for self-ligation. Ligations were carried out for 14–16 h at 14°C.

#### Electroporation of *E. coli* DH10B cells

A 1  $\mu$ l aliquot of the ligation reaction was mixed with 20  $\mu$ l of Electromax DH10B cells (Gibco-BRL) and electroporated at 1.8 kV (BioRad *E. coli* Gene Pulser). Then 1 ml of SOC medium was mixed with the transformed cells and incubated at 37°C for 1 h before plating onto LB agar containing 12.5  $\mu$ g/ml chloramphenicol, 100  $\mu$ g/ml X-gal, and 100  $\mu$ g/ml IPTG. Plates were incubated for 24 h at 37°C, and then transferred to 25°C in darkness for a further 24 h to allow for development of blue/white colonies.

#### Determination of average insert size and arraying of BAC clones

The average insert size in 30–40 recombinant clones from a single ligation was determined prior to arraying of clones for long-term storage as part of the BAC library. BAC DNA was prepared by alkaline lysis of 4-ml 24-h cultures of white colonies from X-gal agar plates. Isolated BAC DNA was digested with *Not*I restriction endonuclease, and loaded onto a 1% agarose CHEF gel in 1 $\times$ TAE buffer. Restriction fragments were separated under the following conditions: a constant temperature of 13°C, a 5–15 s switch time with linear ramping, a voltage gradient of 6 V/cm, 120° included angle, and a run time of 18 h. Ligations that exhibited an average insert size over 80 kb were used for library construction. BAC clones were picked individually into 384 well microtitre plates containing LB-freeze medium (LB broth + 36 mM K<sub>2</sub>HPO<sub>4</sub>, 13.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.7 mM sodium citrate, 400  $\mu$ M MgSO<sub>4</sub>, 6.8 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 4.4% glycerol) with 12.5  $\mu$ g/ml chloramphenicol. Inoculated microtitre plates were grown for 24 h at 37°C, replicated twice, and then frozen at –80°C.

#### High-density filters of BAC clones

Clones from the BAC library were transferred to Hybond N+ nylon membranes (Amersham Pharmacia Biotech) using a Beckman Biomek 2000 Laboratory Workstation. Clones from eight 384 well plates were replicate-transferred to a membrane of 7.8 cm $\times$ 11.9 cm in a 4 $\times$ 4 array, so that each filter contained 3072 BAC clones. Each clone was double spotted in a unique pattern and the position of the clones in the array was designed to be diagnostic of the plate from which the clones originated. The entire BAC library was thus represented on nine filters. Clones arrayed on membranes were grown for 24 h at 37°C on LB-chloramphenicol agar before the bacterial colonies were lysed with alkali to bind the DNA to the membrane.

#### Probe labelling and DNA hybridisation

High-density filters were screened by hybridisation with probes derived from four genes that are known to be single copy genes in *P. infestans*: *calA* encoding calmodulin (Pieterse et al. 1993), *niaA* encoding nitrate reductase (Pieterse et al. 1995), *actB* encoding actin (Unkles et al. 1991), and the *in planta* induced gene IPIDI (Pieterse et al. 1994). Filters were also hybridised with the internal transcribed spacer (ITS) region of the rDNA repeat from *P. infestans* (Cooke et al. 2000). Probes (25 ng) were labelled by

random priming using the High Prime labelling system (Roche). Prehybridisation and hybridisation were carried out at 65°C in a solution containing 7% SDS in 0.5 M sodium phosphate buffer pH 7.2, and 1 mM EDTA. After hybridisation, membranes were washed twice in 5 $\times$ SSPE buffer (Sambrook et al. 1989) at 65°C for 20 min each, and twice in 1 $\times$ SSPE + 0.1% SDS at 65°C for 20 min each. After exposure to Biomax MS X-ray film (Kodak), membranes were stripped for reuse as described by Braithwaite et al. (1991).

#### Pooling of BAC clones

To facilitate screening of the BAC library by PCR-based methods, pools of BAC clones were constructed in “three dimensions”. First, the library was separated into odd and even numbered plates, with 36 plates in each group. Row (A–P) and column (1–24) pools were constructed for each group of plates. One odd numbered plate and one even numbered plate were pooled to form layer pools. Each layer pool contained 768 BAC clones, each row pool contained 864 BAC clones, and each column pool contained 576 BAC clones. Pooled BAC clones were grown at 37°C on LB-chloramphenicol agar. Cells were harvested by scraping colonies from the agar surface in 15 ml of LB broth. BAC DNA from the pooled clones was isolated by alkaline lysis.

#### AFLP DNA fingerprinting

AFLP DNA fingerprinting was performed essentially as described previously (Vos et al. 1995) but with some minor modifications. BAC DNA (50 ng) was digested with *Eco*RI/*Mse*I, and preamplified with non-selective primers. Preamplified DNA was diluted ten-fold in water before amplification with selective primers. After the selective PCR with E+2 and M+2 primers, the liquid in the samples was not evaporated prior to the addition of formamide dye (98% formamide, 20 mM EDTA, with xylene cyanol and bromophenol blue as tracking dyes).

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## Results and Discussion

### Construction of a *P. infestans* BAC library

When constructing BAC libraries, difficulty is generally encountered in routinely obtaining clones with average insert sizes of over 100 kb, despite the fact that the DNA fragments selected for cloning are in the correct size range (Diaz-Perez et al. 1996; Osoegawa et al. 1998; Randall and Judelson 1999). We also experienced this difficulty. Initial attempts to use a single size selection of partially *Hind*III-digested *P. infestans* DNA for construction of the BAC library predominantly yielded inserts averaging less than 70 kb. However, by selecting partially *Hind*III-digested DNA from the size range of 130–250 kb, BAC inserts with an average insert size of 89 kb were obtained (ligation A, Table 1). However, the variation in insert size was large (25–225 kb). Clones estimated to represent 2.7 genome equivalents were picked into 384-well microtitre plates. It was surmised that the smaller DNA fragments were being trapped in the section of the CHEF gel containing the larger restriction fragments, and this was leading to a reduction in the average insert size. Others have used methods such as initial reversal of the polarity of electrophoresis to remove small fragments from the CHEF gel, followed by separation of larger restriction fragments using typical

**Table 1** Average insert sizes and genome representation obtained from the five ligations used in construction of the *P. infestans* BAC library

Ligation	Plates	Cloning enzyme	Average insert size (kb)	Genome representation
A	1–20	<i>Hind</i> III	89	2.7×
B	21–30	<i>Hind</i> III	96.5	1.0×
C	31–33	<i>Hind</i> III	100	0.5×
D	34–40	<i>Hind</i> III	98.5	1.1×
E	41–53	<i>Bam</i> HI	112	2.2×
D	54–72	<i>Hind</i> III	98.5	2.7×

CHEF gel conditions (Osoegawa et al. 1998). We adopted an alternative approach of two size selections to enrich the DNA preparation for larger fragments. The section of the CHEF gel containing the desired size range of fragments was excised, inverted, and electrophoretically fractionated in a second CHEF gel under the same conditions. This had the effect of concentrating the larger DNA fragments into a single band, while the small fragments, which previously co-migrated with larger fragments, were observed as a faint smear below the major band of DNA after staining with ethidium bromide. Ligations using DNA prepared by this method yielded average insert sizes ranging from 97 kb to 100 kb (ligations B–D, Table 1). Some small inserts were still observed in sections of the BAC library prepared in this way. However, they were less numerous and the average insert size was close to, or exceeded, 100 kb. The remaining targeted 7.3-fold genome coverage of the BAC library was established using DNA prepared in this manner.

To avoid bias in genomic representation, two separate restriction endonucleases were used in constructing the BAC library; *Hind*III, which has a recognition sequence that is AT-rich, and *Bam*HI, which has a GC-rich recognition sequence. Clones containing an estimated 2.2 genome equivalents were picked from one ligation of DNA prepared using *Bam*HI as the restriction enzyme (ligation E; Table 1). The average insert size (112 kb) for this section of the BAC library was greater than that from any of the *Hind*III ligation reactions, and the size range was from 80–170 kb.

The complete BAC library, arrayed in 72×384 well microtitre plates, comprises an estimated 10.2 genome equivalents with an average cloned insert size of 98 kb. If ligation A (2.7 genomes, 89 kb average insert size) is excluded from calculations, the average insert size for the *P. infestans* BAC library would be 102 kb.

#### Assessing genomic representation in the BAC library by hybridisation

To ascertain the representation of single copy sequences within the library, high-density filters containing DNA from the BAC clones were screened by hybridisation with probes derived from four genes that are known to be single copy genes in *P. infestans*. Using these genes as probes, four BAC clones containing the *niaA* gene were identified, five for *ipiO*, seven for *actB*, and eight for *calA*.

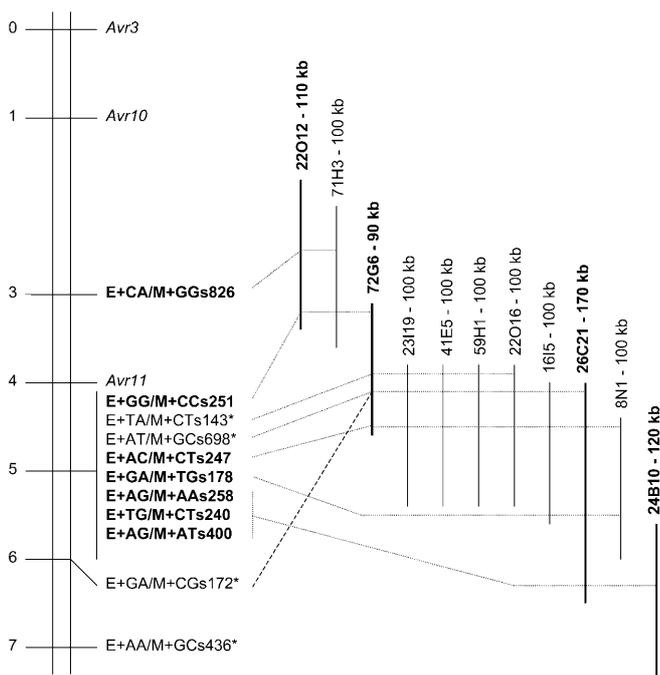
To determine the representation of ribosomal DNA in the BAC library, filters were hybridised with the internal transcribed spacer region (ITS) from *P. infestans* (Cooke et al. 2000). It was estimated that ribosomal DNA is present in at least 2.3% of all clones. Interestingly, no BAC clones containing the ITS region were observed in the portion of the BAC library generated using *Bam*HI as a cloning enzyme.

PCR-amplified fragments of three *P. infestans* mitochondrial genes (*cox1*, *ATP1*, *nad11*; GenBank Accession No. U17009; Paquin et al. 1997) were pooled and used as a probe. No BAC clones were identified which hybridised to this mixture of probes, demonstrating that the size selections of partially digested DNA were successful in excluding the small (38-kb) mitochondrial DNA molecule.

#### Construction of a BAC contig spanning an avirulence locus on Linkage Group VIII

The BAC library was constructed to facilitate positional cloning of *Avr* genes. Recently, Randall and Judelson (1999) described the construction of a *P. infestans* BAC library that comprises clones with an average insert size of 75 kb, representing 4.9 genome equivalents. However, the *P. infestans* isolate used by Randall and Judelson (1999) for BAC library construction has a different genetic background to that of the F<sub>1</sub> individual used here, and may not contain the same *Avr* gene-linked AFLP markers.

Pools of BAC clones were developed to allow screening with AFLP markers linked to *Avr* genes. The genomic region selected for BAC contig construction was a dense cluster of AFLP markers positioned at one end of Linkage Group VIII (Fig. 1). Three avirulence genes (*Avr3*, *Avr10* and *Avr11*) have been genetically mapped to this region (van der Lee et al. 2001). The three-dimensional pools of BAC clones were initially screened with eight AFLP primer combinations that amplify fragments that map to this region (indicated in bold in Fig. 1). Due to the structure of the BAC pools and the identification of multiple positive clones,  $n^3$  potential clone addresses (where  $n$  is the number of positive BAC pools) require a second course of AFLP screening to identify the genuine positives. For example, where five positive clones were identified in one half of the BAC library, 125 potential clones needed to be re-screened. Individual BAC clones from potential clone addresses, identified from positive BAC pools, were



**Fig. 1** Section of linkage group VIII of *P. infestans* (left) (adapted from van der Lee et al. 2001). Indicated are the map positions of AFLP markers used in the first (*bold type*) and second (*asterisks*) screens used to identify and verify BAC clones spanning this genomic region. BAC clones that could be assembled into contigs (not to scale) are indicated as *solid vertical lines* to the right of the linkage group map. Their respective insert sizes are indicated at the top of the solid vertical lines. BAC clones shown in *bold* represent the minimum tiling path across the region. The relative positions of AFLP markers in the BAC contig are shown by *dotted lines*. The position in the BAC contig of AFLP marker E + GA/M + CGs172, which was mapped outside the major cluster of AFLP markers, is indicated by the *dashed line*

re-screened by AFLP fingerprinting to ascertain the addresses of the actual positive BAC clones.

Since dominant PCR-based markers are represented in the BAC library as alleles, it was expected that approximately five positive BAC clones would be identified per polymorphic AFLP marker. Between two and eight positive BAC clones per AFLP marker were identified after the secondary screen. The number of positive BAC clones for AFLP markers is consistent with the calculated genome redundancy in the BAC library, and therefore also consistent with the estimated genome size for *P. infestans*.

Positive BAC clones assembled into contigs, and their corresponding insert sizes, are shown in Fig. 1. From this screen, four BAC clones were observed to contain more than one AFLP marker. Clone 26C21 was observed to contain five of the markers screened. BAC clones were further analysed for specific AFLP fragments using four additional primer combinations from the cluster of markers at the end of LG VIII. This further confirmed the preliminary contig of BAC clones and joined BACs 72G6, 71H3, and 22O12 to the larger part of the contig. By linkage mapping, marker

E + GA/M + CGs172 is located at 6 cM on the linkage map. However, this marker was determined to be physically located within the BAC contig, amplifying from the same BAC clones as E + AT/M + GCs698. This situation may have arisen if one F<sub>1</sub> individual in the mapping population had been mis-scored for this marker. In contrast, marker E + AA/M + GCs436 could not be amplified from any of the BAC clones in the contig, demonstrating that the contig does not extend as far as this marker.

BAC clones were digested with restriction enzymes, individually and in combination, to determine the overlap between clones and form contigs spanning the genomic region. By this approach, it was determined that all but two of the identified BAC clones formed a single contig (Fig. 1). Both of the unassembled clones were identified using the EAA + /M + CTs239 marker. Since the AFLP markers in this region are densely clustered, and therefore difficult to order accurately, it is likely that these two BAC clones lie further away from the clones in the contig. This could be confirmed by testing more markers in this region, or mapping the ends of these BAC clones.

Although the contig contains 11 BAC clones, the minimum tiling path across the region is represented by only four clones: 22O12, 72G6, 26C21, and 24B10 (Fig. 1). Given the size of these clones and the clear overlap between them, as revealed by both AFLP and restriction digestion analyses, the minimum contig contains less than 490 kb of DNA (the sum of BAC inserts in the minimum tiling path).

The genetic position of *Avr11*, as determined by van der Lee et al. (2001) suggests that this gene should be contained within the BAC contig. Mapping of the ends of the BAC contig on the AFLP linkage map should reveal whether or not the BAC contig also contains *Avr3* and *Avr10*. If *Avr3* and *Avr10* cannot be demonstrated to lie within the BAC contig, then a chromosome walk will be required to extend the BAC contig to encompass these loci.

#### Concluding remarks

In this report we have presented a physical map of an avirulence locus in *P. infestans*, and this represents an important step towards the positional cloning of the first *P. infestans* *Avr* gene. Recently, Randall and Judelson (1999) reported successful transformation and stable integration of entire BACs into the genome of *P. infestans*. This approach could be used to confirm the presence of *Avr* genes within the BAC contig presented here. Transformation of entire BACs containing markers flanking *Avr* loci into *P. infestans* isolates lacking the *Avr* allele should result in a change in phenotype from virulent to avirulent on a potato line carrying the corresponding resistance gene. In this instance, the four BAC clones forming the minimum tiling path across the region would be used to determine which, if any, contain

*Avr* genes. Subcloning of the BACs, followed by further transformation experiments could then be used to identify the fragment of genomic DNA containing an *Avr* gene. Alternatively, the entire BAC contig could be sequenced, ORFs predicted, and each ORF tested by transformation for function as an *Avr* gene.

Further to positional cloning of genes, the BAC library will also serve as a valuable resource for future genomic studies of *P. infestans* and other plant pathogenic oomycetes. For *P. infestans*, the existence of a genetic linkage map (van der Lee et al. 1997) and a set of expressed sequence tags (ESTs) (Kamoun et al. 1999) would make it possible to integrate transcriptional, physical and genetic maps. Moreover, the BAC library will underpin efforts directed at understanding genome structure in *P. infestans* through comparisons with genome structure in other oomycetes, such as the soybean pathogen *P. sojae* (Mao and Tyler 1991; Whisson et al. 1995) and *Peronospora parasitica*, a pathogen of the model plant *Arabidopsis thaliana* (Voglmayr and Greilhuber 1998; Rehmany et al. 2000). Such efforts will lead to an enhanced understanding of these important plant pathogens and support the development of novel strategies for their control.

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## CHAPTER 5

# CHROMOSOMAL DELETION IN ISOLATES OF *PHYTOPHTHORA* *INFESTANS* CORRELATES WITH VIRULENCE ON *R3*, *R10*, AND *R11* POTATO LINES



# Chromosomal Deletion in Isolates of *Phytophthora infestans* Correlates with Virulence on *R3*, *R10*, and *R11* Potato Lines

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In *Phytophthora infestans*, a cluster of three dominant avirulence genes is located on the distal part of linkage group VIII. In a mapping population from a cross between two Dutch field isolates, probe M5.1, derived from an amplified fragment length polymorphism (AFLP) marker linked to the *Avr3-Avr10-Avr11* cluster, hybridized only to DNA from the parent and F1 progeny that is avirulent on potato lines carrying the *R3*, *R10*, and *R11* resistance gene. In the virulent parent and the virulent progeny, no M5.1 homologue was detected, demonstrating a deletion on that part of linkage group VIII. *P. infestans* is diploid, so the avirulent strains must be hemizygous for the region concerned. A similar situation was found in another mapping population from two Mexican strains. The deletion was also found to occur in many field isolates. In a large set of unique isolates collected in The Netherlands from 1980 to 1991, 37% had no M5.1 homologue and the deletion correlated strongly with gain of virulence on potato lines carrying *R3*, *R10*, and *R11*. Also, in some old isolates that belong to a single clonal lineage (US-1) and are thus highly homogenous, deletions at the M5.1 locus were detected, indicating that this region is unstable.

*Additional keywords:* late blight, oomycetes, plant pathogen.

*Phytophthora infestans* causes late blight on both potato and tomato and is one of the most devastating plant pathogens worldwide. Attempts to control late blight by introducing resistance into potato cultivars have been largely unsuccessful. The level of broad-spectrum or horizontal resistance in commercial cultivars is insufficient to control the pathogen, and the use of single major resistance genes (*R* genes) has been hampered by the complex race structure of most *P. infestans* populations (Drenth et al. 1994; Schöber 1975; Shattock et al. 1977). In the last 2 decades, late blight reemerged as a serious threat to potato production. In the late 1970s, a new *P. in-*

*festans* population migrated from Mexico, the center of diversity, to Europe. This resulted in a worldwide displacement of the existing population, which was dominated by a single clonal lineage of the A1 mating type, termed US-1. The new populations are genetically more diverse and consist of A1 and A2 mating-type isolates (Drenth et al. 1994; Fry et al. 1992). Since *P. infestans* is heterothallic, the occurrence of both mating types led to the establishment of sexual reproduction and recombination, and it is likely that this caused the evolution of more aggressive isolates now found in many populations (Day and Shattock 1997; Flier and Turkensteen 1999). In order to elucidate the complexity of race structure and the evolution and selection of new races of *P. infestans*, we study the genetics of *R* gene-specific virulence.

*P. infestans* belongs to the oomycetes; its somatic hyphae are diploid and meiosis occurs just before mating in well-differentiated oogonia and antheridia. Genetic analysis of *P. infestans* is hampered by the lack of well-defined laboratory strains and mutants (Judelson 1996a). Moreover, sexual progeny especially from back crosses and sibling crosses is difficult to generate. In recent years, various types of molecular markers have been exploited to study inheritance in *P. infestans* (Carter et al. 1999; Goodwin et al. 1992; van der Lee et al. 1997). Segregation of markers in sexual progeny appears to be largely Mendelian. Nevertheless, deviations are found, mainly due to variation in ploidy levels. The occurrence of triploid, trisomic, or aneuploid progeny and hemizygous regions is not uncommon (Carter et al. 1999; Judelson 1996b; van der Lee et al. 1997; T. van der Lee and F. Govers, *unpublished data*). This phenomenon is consistent with the variable amounts of nuclear DNA content often found in *P. infestans* isolates (Tooley and Therrien 1987).

The variation in ploidy levels hampers the analysis of inheritance of *R* gene-specific virulence in *P. infestans* without linked molecular markers. Previously, we identified markers linked to six avirulence genes in *P. infestans*. Interestingly, three avirulence genes, *Avr3*, *Avr10*, and *Avr11* cluster on a distal part of linkage group VIII (van der Lee et al. 2001). In order to isolate and characterize the *Avr3-Avr10-Avr11* gene cluster, we have cloned the linked amplified fragment length polymorphism (AFLP) markers and used these as probes on Southern blots containing DNA from the parental lines of a mapping population that was used for the construction of a molecular genetic linkage map (van der Lee et al. 1997). One cloned AFLP marker hybridized to DNA from the parent that

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is avirulent on potato lines carrying resistance gene *R3*, *R10*, or *R11*, but not to DNA from the parent that is virulent on these lines. In this paper, we study to what extent lack of this AFLP marker correlates with virulence. For this purpose, not only were two F1 mapping populations screened but also a large set of field isolates with known virulence patterns. The data show that the majority of the field isolates that lack the marker are indeed virulent on potato lines carrying resistance genes *R3*, *R10*, and *R11*. The significance of this finding for the evolution of virulent races of *P. infestans* will be discussed.

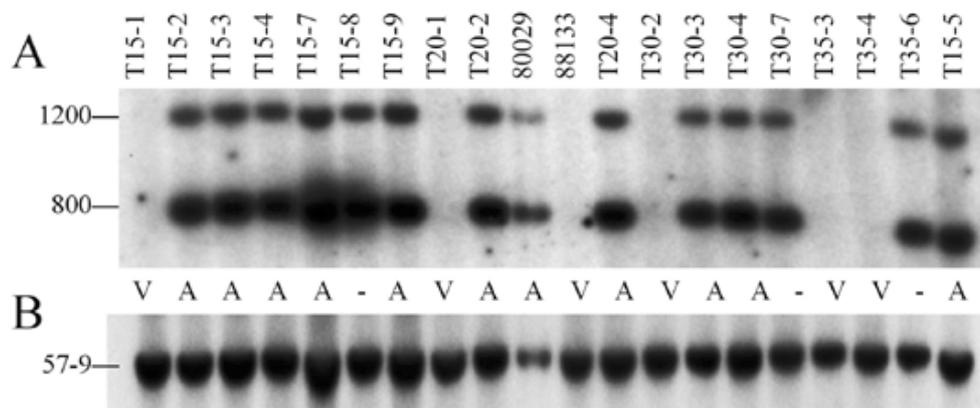
## RESULTS

### AFLP markers linked to the *Avr3-Avr10-Avr11* cluster on linkage group VIII.

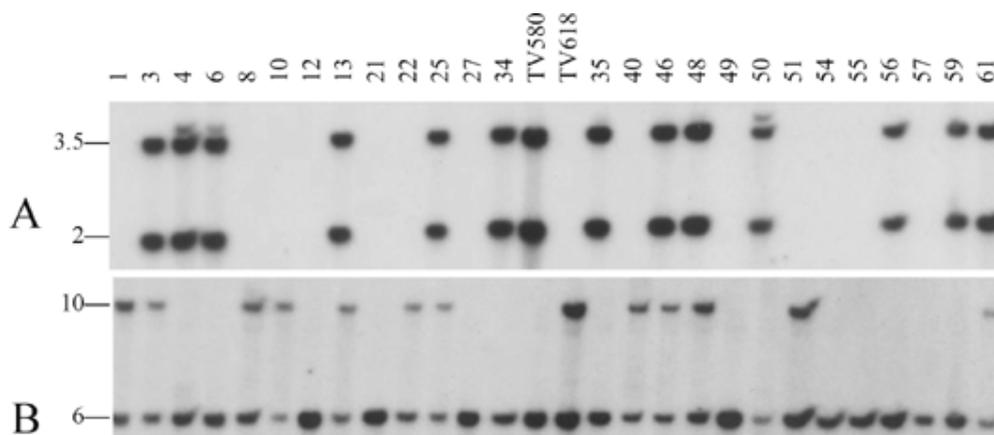
Fifteen AFLP markers tightly linked to the *Avr3-Avr10-Avr11* cluster were identified using bulked segregant analysis

(BSA) (van der Lee et al. 2001). All identified markers, i.e., 15 out of 25,000 fragments analyzed in the BSA, were in coupling phase with avirulence, while markers in repulsion phase in over 1,000 random markers were lacking. The deviation from the expected 1:1 ratio between markers in coupling and repulsion phases could be due to the absence of a homologous region on the other chromosome.

Twelve of the fifteen linked AFLP markers were cloned and used as probes on Southern blots containing genomic *P. infestans* DNA digested with *EcoRI*. Since the AFLP fragments were generated by polymerase chain reaction (PCR) amplification of *EcoRI-MseI* restriction fragments, they contain no internal *EcoRI* restriction site. Thus, fragments that are single copy in the genome should show one or, if the two homologous alleles are polymorph, two hybridizing fragments in the parental isolates. Nine of the twelve cloned AFLP markers, however, hybridized to several restriction fragments of differ-



**Fig. 1.** Segregation of marker M5.1 in the F1 progeny of *Phytophthora infestans* cross 71 (80029 × 88133). Autoradiographs of Southern blots containing total DNA from *P. infestans* isolates digested with *EcoRI* and hybridized with **A**, probe M5.1 and **B**, DNA fingerprint probe RG57. Virulence phenotypes on *R3*, *R10*, and *R11* potato plants are indicated by A for avirulent, V for virulent, or – for unknown. The sizes of the fragments (bp) are indicated on the left. The lanes contain DNA from the parental lines (80029 and 88133) and their F1 progeny (indicated by numbers with a prefix T). Two fragments in the avirulent parent, 80029, and the avirulent progeny hybridize to M5.1, but no homologous sequence is detected in the virulent parent, 88133, or the virulent progeny. Hybridization of the same blot with the multicopy probe RG-57 demonstrates that each lane contains DNA. **B**, Only one of the fragments (fragment 9) hybridizing to RG57 is shown.



**Fig. 2.** Segregation of marker M5.1 in the F1 progeny of *Phytophthora infestans* cross 68 (TV580 × TV618). Autoradiographs of Southern blots containing total DNA from *P. infestans* isolates digested with *BamHI* and **A**, hybridized with probe M5.1 and **B**, *calA*, a *P. infestans* gene encoding calmodulin. The sizes of the fragments (kb) are indicated on the left. The lanes contain DNA from the parental lines (TV580 and TV618) and their F1 progeny (indicated by numbers). M5.1 hybridizes to two fragments in parent TV580 that is avirulent on *R3* carrying potato lines and in part of the F1 progeny. In the other lanes, including the one containing DNA from the virulent parent TV618, no M5.1 homologue is detected. Hybridization of the same blot with a *calA* probe demonstrates that each lane contains DNA. This probe detects a polymorphism in the two parental lines that segregates in the F1 progeny.

ent lengths, suggesting that the cloned fragments contain repetitive DNA. Also, several of the cloned AFLP markers hybridized to more fragments in the DNA of the avirulent parent (strain 80029) as compared with the DNA of the virulent parent (strain 88133) (data not shown). Among these was marker M7.1, derived from the most distal AFLP marker on this linkage group, i.e., AE+CA/M+GGs826. M7.1 hybridized to up to seven fragments of different length. Several weaker hybridizing fragments were not polymorphic between the parental isolates and no intensity difference was observed, but two strongly hybridizing fragments were found in the DNA of the avirulent parent that were lacking in the virulent parent.

Two markers, M11.1 derived from marker AE+CT/M+ATs306 and M12.1 derived from marker AE+CT/M+TGs308, detected a single-copy *EcoRI* fragment of the same size (data not shown). Possibly, the two *EcoRI-MseI* markers are situated internally on the same *EcoRI-EcoRI* fragment. Neither M11.1

nor M12.1 revealed an *EcoRI* fragment length polymorphism between the two parental isolates.

One cloned AFLP fragment, designated M5.1 and derived from marker AE+AT/M+AGs400, hybridized to an 800- and a 1,200-bp *EcoRI* fragment of the avirulent parent 80029 but, even at low stringency hybridization conditions (0.5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] at 55°C) and long exposure times, no hybridizing fragments were detected in the virulent parent 88133 (Fig. 1A). Lowering the hybridization and washing temperature from 65 to 55°C did not change the hybridization pattern. The hybridization signal of the 800-bp fragment was stronger than that of the 1,200-bp fragment, suggesting that M5.1 is derived from the 800-bp fragment and that the 1,200-bp fragment contains a homologue. When M4.1, one of the multicopy probes derived from AFLP marker AE+AG/M+AAAs258, was used as probe for Southern blot hybridizations, an 800-bp fragment was de-

**Table 1.** Occurrence of the M5.1 homologue in field isolates of *Phytophthora infestans*<sup>a</sup>

Isolate <sup>b</sup>	Mating type	RG57 genotype <sup>c</sup>	M5.1	Virulence <sup>d</sup>				
				R1	R3	R4	R10	R11
80029	A1	01001000001011010001010101	+	A	A	V	A	A
81197	A2	11001010000011110011110101	-	V	V	V	V	V
84044	A1	11101011001011000011110101	+	V	V	V	V	V
85005	A1	10101110001011010101110101	+	V	A	V	A	A
85025	A2	11001101001011100001010101	-	V	V	V	V	V
85026	A2	11001101111011100001010101	+	V	V	V	V	V
85027	A2	10001101111011100001010101	+	V	V	V	V	V
85206	A1	11101111101011110011110101	+	V	V	V	V	-
86057	A2	10001000000011110011110101	-	A	A	V	A	V
87001	A2	01001000001011110001110101	+	A	A	V	A	A
87010	A2	10001100001011100011110101	-	V	A	V	V	V
87013	A1	11101111101011110001110101	+	V	V	V	-	-
87030	A2	11101010001011100011110101	-	A	A	V	V	V
87032	A2	11000100000011110001010101	-	A	V	A	A	V
87034	A2	10101010000011100001100101	+	V	-	V	V	V
87073	A2	1010111100011110000100101	+	A	A	V	A	A
87086	A2	11001000001011110011110101	+	V	A	V	A	A
87110	A2	10001000001011100001110101	+	A	V	V	A	V
87125	SF <sup>e</sup>	11101111001011010001110101	-	V	V	V	V	V
87134	A2	10001000001011110011111001	+	V	A	A	A	A
87148	A2	11001000001011101001010101	-	V	A	V	A	V
87174	SF <sup>e</sup>	10101110001011100001100101	+	A	V	A	V	V
87177	A1	10101110001011000011110101	-	V	V	V	V	A
88013	A2	10001000101011101001100101	+	V	V	A	V	V
88014	A2	10101010001011100001100101	+	V	V	A	A	V
88034	A2	10001001000011110011110101	+	V	A	V	A	A
88046	A1	11101110101011110011110101	-	V	V	V	V	V
88051	A2	11101111001011110011110101	-	V	V	V	V	V
88052	A2	11101111000011110001100101	-	A	V	V	V	V
88055	A2	01001100101011110011110101	+	A	V	V	V	V
88067	A1	10101010001011101011100101	+	A	V	V	-	-
88133	A2	10101011001011100111110101	-	V	V	A	V	V
88148	A2	10101111000011100001100101	-	V	V	V	V	V
88165-01	A1	10101111101011110011100101	+	V	V	V	V	V
88175	A2	10001000001011110001110101	-	V	V	V	V	V
88212	A2	10101010001011100101100101	-	V	V	A	A	V
89019	A2	11101110001011110001110101	+	A	A	V	A	A

(continued on next page)

<sup>a</sup> The isolates were collected in The Netherlands from 1980 to 1991. Their mating type, RG57 DNA fingerprinting pattern, and virulence phenotype toward potato lines carrying resistance gene *R1*, *R3*, *R4*, *R10*, or *R11* are listed.

<sup>b</sup> First two numbers refer to the year of isolation.

<sup>c</sup> DNA fingerprinting pattern refers to bands 1, 2, 3, 4, 5, 6, 7, 8, 9, 9a, 10, 12, 13, 14, 14a, 16, 17, 18, 19, 20, 21, 22, 23, 24, 24a, and 25 hybridizing to probe RG57 as defined by Goodwin and associates (1992) and Drenth and associates (1993). The binary code 1 and 0 represents presence and absence, respectively.

<sup>d</sup> Virulence on potato plants carrying the *R* gene listed. V = virulent, A = avirulent, and - = unknown

<sup>e</sup> SF = self fertile.

tected in parent 80029 next to seven other hybridizing fragments (data not shown). Also, in this case, the 800-bp *EcoRI* fragment was absent in the virulent parent and it was the strongest hybridizing fragment in the avirulent parent, indicating that this is the *EcoRI-EcoRI* fragment from which the marker is derived. Since the markers M4.1 and M5.1 showed clear polymorphism between the parental isolates, they were used for further studies on the sexual progeny of the cross 80029 × 88133 (cross 71).

#### Segregation of marker M5.1 in mapping populations.

M5.1 hybridized to an 800- and a 1,200-bp *EcoRI* fragment of parent 80029, the avirulent parent from cross 71 in which the initial AFLP marker was identified. The two fragments cosegregated in the F1 progeny of cross 71, showing that the two homologues detected in the 80029 parent are not allelic but reside on the same chromosome (Fig. 1). The restriction fragment length polymorphism (RFLP) markers generated

with M5.1 cosegregated with the cluster of *Avr3-Avr10-Avr11* linked markers including marker AE+AT/M+AGs400. Only DNA from the avirulent progeny hybridized to M5.1, demonstrating that only F1 progeny avirulent on potato lines carrying *R3*, *R10*, and *R11* contain the homologues (Fig. 1A). The 800-bp fragment identified upon hybridization with M4.1 also cosegregated with markers tightly linked to the *Avr3-Avr10-Avr11* cluster in the progeny of cross 71 (data not shown).

To investigate whether M5.1 segregates in a similar way in other crosses, we analyzed an unrelated cross between two Mexican isolates, TV580 × TV618 (cross 68, generated and kindly provided by L. J. Spielman and W. E. Fry, Cornell University; Goodwin et al. 1992). The same two *EcoRI* fragments were present in the parent that is avirulent on *R3* plants (TV580) and absent in the parent virulent on *R3* (TV618). Hybridization of M5.1 to *BamHI*-digested DNA again revealed two fragments in TV580, but no hybridization was detected in TV618 (Fig. 2). Also in cross 68, M5.1 segregates (Fig. 2).

Table 1. (continued from preceding page)

Isolate <sup>a</sup>	Mating type	RG57 genotype <sup>b</sup>	M5.1	Virulence <sup>c</sup>				
				<i>R1</i>	<i>R3</i>	<i>R4</i>	<i>R10</i>	<i>R11</i>
89038	A2	01001000000011110001010101	+	A	V	V	V	V
89056	A2	10101110101011110111110101	-	A	V	V	V	V
89061	A1	10101111101011110111110101	+	V	V	V	V	A
89070	A2	11001100101011110001010101	-	V	V	V	V	V
89075	A2	11101100101011111001010101	-	V	V	V	A	A
89076	A1	1010111101011110001110101	-	V	V	V	V	V
89081	A1	0110101010101111011110101	-	A	V	V	A	V
89140-01	A1	11111110101011110011110101	+	V	V	V	V	V
89140-02	A1	11111111101011110011110101	+	V	V	V	V	V
89140-07	A1	10111011001011110001100101	-	V	V	V	V	V
89140-09	A2	10011001001011100011110101	-	V	V	V	V	V
89140-15	A1	10111010001011110001100101	-	V	V	V	V	V
89141-02	A1	11111011001011110011110101	-	V	V	V	V	V
90041	A1	11101111001011110001110101	-	V	V	V	V	V
90056-02	A2	1110111001101101010000110101	+	V	A	V	V	V
90056-03	A2	10001100001011000010100101	-	A	V	A	V	V
90062	A2	10101110001011010011100101	+	A	A	V	V	V
90080	A2	11101011001011110011110101	+	A	A	V	A	A
90083	A2	00101010101011110011100101	+	V	A	V	A	A
90101	A1	10101110001011110011101101	+	A	V	A	V	V
90111	A2	11001001101011000011110101	+	A	A	V	V	V
90121	A2	11001000100011000001110101	+	V	A	V	A	A
90124	A2	11101010001011010001010101	+	V	V	V	V	V
90125	A1	11101010001011110001110101	-	A	V	V	-	V
90127	A2	10101010001011000011100101	-	V	V	V	A	V
90128	A2	10001000101011100001100101	+	V	V	V	V	V
90137	A1	11000100001011100010110011	+	V	V	V	V	V
90149	A2	10001100100011110001100101	+	V	V	V	A	V
90190	A1	10001010000011100001100101	-	V	V	V	V	V
90196	A2	10101011001011110001100101	+	A	A	V	A	A
90208	A2	10101010101010110001100101	+	V	V	V	A	V
90209	A1	11001000001011110001110101	-	V	V	V	V	V
90215	A1	10101111001011110011100101	+	V	V	V	A	A
91001	A2	10101011010011111001100101	+	A	A	A	A	A
91002	A2	10101011101011110001100111	+	A	A	A	A	A
91004	A2	101010110100111110001100101	+	V	V	A	V	V
91005	A2	11001000111011110001110101	+	V	A	V	A	A
91006	A2	11001000001011101001110111	+	V	A	V	A	A
91007	A2	10101010011011110011100101	+	V	V	V	A	V
91008	A1	1010111111011110011100101	+	V	V	V	V	V
91009	A1	10001001101011101011100101	+	V	V	A	V	V
91010	A2	11101010111011110001110101	+	V	V	V	A	A
91011	A2	10101010001011110011110011	+	A	V	V	A	V
91012	A2	10101010111011110011110101	+	A	V	V	V	-
91014	A1	10101110001011110011110101	+	V	V	V	V	V
91015	A1	11101011001011110101111111	+	V	A	V	A	A

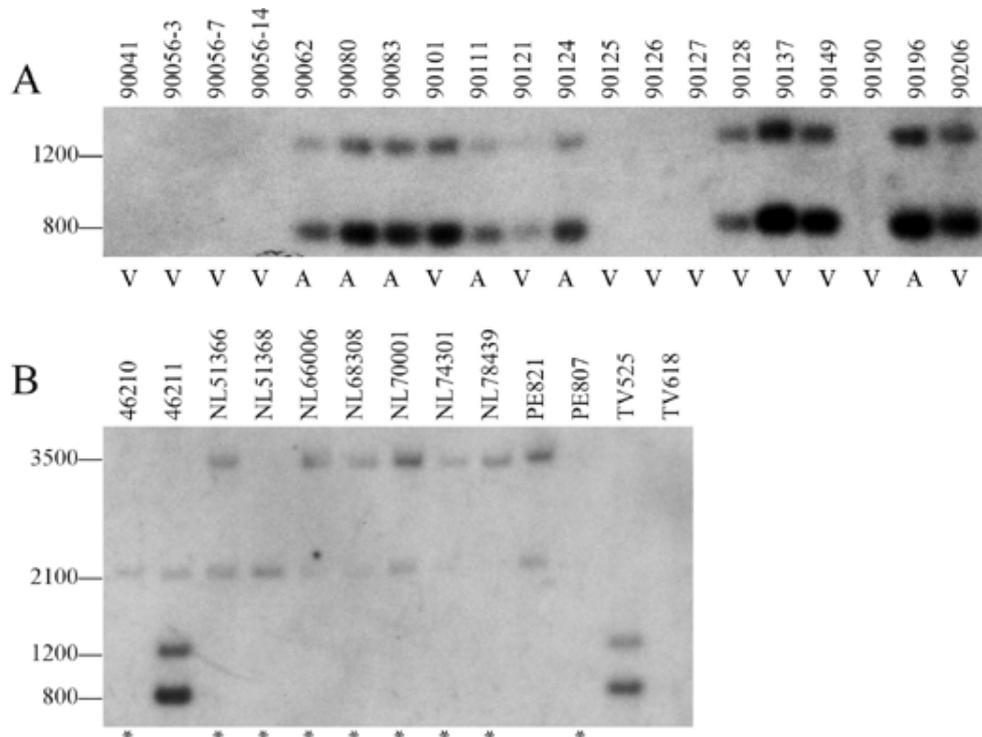
The virulence of isolates TV580 and TV618 on *R10* and *R11* plants is not known, but in the progeny of cross 68, the two fragments generated with M5.1 cosegregated with AFLP markers that are mapped close to the telomere on linkage group VIII in cross 71, where the *Avr3-Avr10-Avr11* cluster is located (van der Lee et al. 2001).

### Occurrence of marker M5.1 in field isolates.

The parents of cross 71 are field isolates that were collected in The Netherlands in 1980 (80029) and 1988 (88133). To investigate the occurrence of marker M5.1 in Dutch field populations, we selected a set of 83 field isolates collected between 1980 and 1991. The set is a mixture of A1 and A2 mating-type isolates and includes the parents of cross 71. As was determined previously (Drenth et al. 1994), each of these isolates shows a unique hybridization pattern with the multilocus DNA fingerprint probe RG57 (Table 1). Hybridization of *EcoRI*-digested DNA of these field isolates with probe M5.1 either yielded the 800- and 1,200-bp fragments (52 or 63% of the isolates) or no hybridization signal was seen (31 or 37% of the isolates) (Fig. 3A, Table 1). As the region is hemizygotously present in the avirulent parents of cross 71 and cross 68, stronger intensities of hybridization to the 800- and 1,200-bp fragments might reveal homozygosity for the M5.1 locus in some of the field isolates. However, there were no indications for the occurrence of isolates homozygous at the M5.1 locus; differences in observed signal intensities were also seen with

other probes on the same blots, e.g., RG57, and were due in all cases to differences in the amount of DNA loaded on gel.

Isolates collected before the second worldwide migration of *P. infestans* (before 1980) are genetically highly similar (Drenth et al. 1993, 1994; Fry et al. 1992). When fingerprinted with the fingerprint probe RG57, most of these so-called 'old' isolates show an identical or nearly identical genotype that has been designated US-1. With AFLP DNA fingerprinting, some genotypic variation was found, albeit limited (Kamoun et al. 1998). We analyzed several Dutch representatives of the US-1 clonal lineage for the presence of M5.1 and found they showed a different hybridization pattern when compared with the 'new' isolates. The 800- and 1,200-bp fragments were absent, but a 2,100-bp or a 3,500-bp fragment, or both, was present (Fig. 3B). The hybridization signal was much lower when compared with the signals obtained with the 800- and 1,200-bp fragments, indicating that the 2,100- and 3,500-bp fragments are only distantly related to M5.1. In a Peruvian US-1 type isolate (PE-807, Fig. 3B), no hybridization was detectable. The 2,100- and 3,500-bp fragments were found in another isolate from Peru (PE821), but that isolate has an RG57 genotype other than US-1. In contrast, a non-US-1 type isolate from Mexico (TV525; Fig. 3B) has the 800- and 1,200-bp fragments similar to the new isolates from The Netherlands and to TV580, the A1 parent from cross 68. As in the Mexican isolates and the new isolates from The Netherlands, as well as in the US-1 isolates,



**Fig. 3.** Occurrence of marker M5.1 in field isolates of *Phytophthora infestans*. Autoradiographs of Southern blots containing total DNA from *P. infestans* field isolates digested with *EcoRI* and hybridized with probe M5.1. **A**, Dutch field isolates collected in 1990. Virulence phenotypes on *R3*, *R10*, and *R11* potato plants are indicated by A for avirulent, V for virulent, or - for unknown. Isolates 90056-3, 90056-7, and 90056-14 have identical RG57 genotypes (only 90056-3 is listed in Table 1). Similarly, isolates 90125 and 90126 are identical (only 90125 is listed in Table 1). The RG57 genotype of isolate 90206 is identical to that of isolate 88165-1 (listed in Table 1). **B**, Various field isolates from either unknown origin (46210 and 46211), The Netherlands (NL), Peru (PE), or Mexico (TV; Toluca Valley). The asterisk (\*) indicates US-1 or US-1-related isolates. The sizes of the hybridizing fragments (bp) are indicated on the left.

polymorphism at the M5.1 locus seems to be due to deletions; in some cases, either the 2,100-bp fragment or the 3,500-bp fragment is lacking. In other cases, the M5.1 locus is completely absent. A complicating matter is isolate 46211. Isolates 46210 and 46211 were deposited in the American Type Culture Collection in 1946 but their origin is unknown. Isolate 46210 resembles the other US-1 type isolates, but in 46211, the 2,100-bp fragment is present in combination with the 800- and 1,200-bp fragments (Fig. 3B).

**Correlation between absence of a M5.1 homologue and virulence on potato lines carrying resistance genes *R3*, *R10*, and *R11*.**

Since the absence or presence of bands hybridizing to M5.1 matched the segregation of AFLP marker AE+AT/M+AGs400 in cross 71, and since this AFLP marker is tightly linked to the *Avr3-Avr10-Avr11* cluster (van der Lee et al. 2001), polymorphism of M5.1 in field isolates opens the possibility of studying its correlation with (a)virulence in field isolates toward potato lines carrying *R3*, *R10*, and *R11*.

The set of 83 field isolates was previously tested for virulence (Drenth et al. 1994) on the same differential set of potato genotypes as was used for mapping the *Avr3-Avr10-Avr11* cluster in cross 71 (van der Lee et al. 2001). Combination of the virulence data and M5.1 hybridization data (Table 1) showed a correlation between virulence on *R3*, *R10*, or *R11* potato plants and absence of the M5.1 homologue (Table 2). Such a correlation is not found with virulence on *R1* and *R4* plants. The  $\chi^2$  distribution resulted in *P* values varying from *P* < 0.001 for virulence on *R11* to *P* < 0.02 for virulence on *R3* and *R10* (Table 2). Combining (a)virulence on all three differentials with the absence of marker M5.1 resulted in a corresponding *P* value of *P* < 0.0005. This correlation is mainly based on the complete absence of avirulent isolates that lack a homologue for marker M5.1. In contrast, the presence of marker M5.1 had no, or limited, predictive value for a virulent phenotype. Many isolates (nearly 50%) that have a homologue for M5.1 were still virulent on potato lines carrying *R3*, *R10*, and *R11*. The relation of the deletions in the US-1 genotypes with virulence toward potato lines with *R3*, *R10*, and *R11* is difficult to determine. The number of available US-1 isolates is limited and many of them are no longer pathogenic enough to perform reliable virulence tests on a differential set.

**DISCUSSION**

In the search for AFLP markers genetically linked to the *Avr3-Avr10-Avr11* cluster, we expected an even distribution of markers in coupling and repulsion phases with the *Avr* genes. Instead, all 15 tightly linked markers identified in cross 71 were in coupling phase with avirulence (van der Lee et al. 2001). We hypothesized that a homologous region for this avirulence locus might be lacking on one of the two homologous chromosomes. Indeed, we identified a region on the distal part of linkage group VIII that is only present on the chromosome that carries the dominant *Avr* genes. We cloned 12 linked AFLP markers and used them as RFLP probes for genomic Southern blots. This would enable identification of all alleles of the locus. Most of the cloned AFLP markers hybridized to multicopy fragments in genomic DNA of *P. infestans*. With some markers, including M7.1 representing the most distal marker on this linkage group, additional fragments were detected in the DNA of the avirulent parent of cross 71 as compared with DNA from the virulent parent. However, since these additional fragments might be allelic to other fragments at different locations in the genome, these data are inconclusive for proof of a deletion. Fortunately, three probes detected single-copy fragments and one, probe M5.1, hybridized only to DNA from the avirulent parent and the avirulent progeny of cross 71. This clearly showed that this chromosome contains a region for which no homologue is present, neither on the homologous chromosome in the avirulent parent nor on the homologous chromosomes of the virulent parent (Fig. 4). The same situation was found in the parents and progeny of a second unrelated cross, cross 68. Since a high proportion of this region contains repetitive DNA sequences, the size of the deletion is difficult to determine by Southern blot hybridization. However, an indication of the size of the deletion may be obtained from the number of AFLP markers that was generated. In the AFLP-based BSA, around 25,000 fragments were screened for linkage to the *Avr3-Avr10-Avr11* cluster (van der Lee et al. 2001). Given an expected genome size of 250 Mb, on average, one AFLP fragment was generated every 10 kb. Due to the deletion, all unique AFLP fragments in the region would be polymorphic and, therefore, the 15 tightly linked AFLP markers would represent around 150 kb. If all AFLP fragments in this region were polymorphic, this would also mean that the region should be highly saturated with markers.

**Table 2.** Correlation between virulence of 83 Dutch *Phytophthora infestans* field isolates toward potato lines carrying resistance genes *R3*, *R10*, and *R11*, either alone or together, and the presence of the M5.1 homologue<sup>a</sup>

<i>R</i> gene	<i>N</i> <sup>b</sup>	M5.1+		M5.1-		<i>P</i> <sup>c</sup>
		VIR	AVR	VIR	AVR	
<i>1</i>	83	34	18	23	8	0.402
<i>3</i>	82	31	20	27	4	0.011
<i>4</i>	83	43	9	27	4	0.593
<i>10</i>	80	25	25	23	7	0.018
<i>11</i>	79	28	20	29	2	0.0007
<i>3, 10, 11</i> <sup>d</sup>	55	18	17	20	0	0.00018

<sup>a</sup> For the isolates containing (M5.1+) or lacking (M5.1-) M5.1, the numbers of isolates avirulent (AVR) and virulent (VIR) on plants carrying the indicated *R* gene are listed.

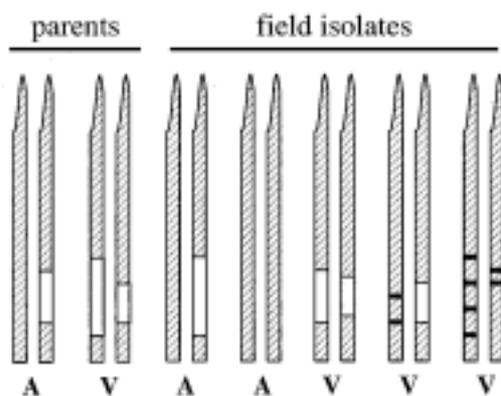
<sup>b</sup> Number of isolates for which the virulence phenotype is known.

<sup>c</sup> *P* value for the significance of deviation from the expected ratio using a  $\chi^2$ -test (3 df) calculated using the probabilities for virulence and the presence of marker M5.1 over the total number of genotypes.

<sup>d</sup> Combined virulence or avirulence on potato lines carrying *R3*, *R10*, and *R11*; only those isolates in which all three phenotypes are either avirulent or virulent are included.

Indeed, hybridization with the cloned AFLP markers suggests that by BSA in two independent complete genomic screens we have landed twice on the same *EcoRI* fragment. M4.1 and M5.1 hybridize to the same *EcoRI* fragment, and also M11.1 and M12.1 hybridize to *EcoRI* fragments of the same size and likely to be identical. In contrast to M5.1, M11.1 and M12.1 are present in both the virulent and the avirulent parent, which might imply that there are more deletions in the region or that the deletion is smaller than calculated above.

In the late 1970s to early 1980s, Europe was invaded by a new population of *P. infestans* (Drenth et al. 1994). The new population shows a large genetic variation but no distinctive genetic clusters (Kamoun et al. 1998; Zwankhuizen et al. 2000). It is assumed that sexual reproduction plays an important role in the continuous appearance of new genotypes in *P. infestans* populations in The Netherlands (Drenth et al. 1994; Zwankhuizen et al. 2000). Despite the large genetic variation, virulence toward particular *R* gene combinations is observed more frequently than expected on the basis of random distribution. This is especially true for virulence on *R10* and *R11* plants, and this might point to a common origin for these virulence phenotypes. The clustering of *Avr3*, *Avr10*, and *Avr11* on linkage group VIII suggests a single region determining avirulence toward a number of *R* genes. We observed a strong correlation between the absence of marker M5.1 and the virulence of *P. infestans* field isolates on potato lines carrying *R3*, *R10*, and *R11*. This correlation was not observed with virulence on potato lines carrying *R1* and *R4* and it suggests a similar organization or regulation, or both, of the *Avr3-Avr10-Avr11* cluster in many field isolates. The strong correlation is due to the low number of isolates that lack M5.1 and are avirulent (two for *R11*, four for *R3*, and seven for *R10*). In fact, these numbers are close to the error rates that occur in the virulence testing on the standard differential set. Isogenic *R* gene-containing potato lines are not available, so the standard set is composed of potato lines with the various *R* genes in different genetic backgrounds. Especially *R10* is difficult to score.



**Fig. 4.** Hypothetical model of the distal part of the two homologues of linkage group VIII of *Phytophthora infestans* in the parents of two crosses and in field isolates. Virulence phenotypes on *R3*, *R10*, and *R11* potato plants are indicated by A for avirulent and V for virulent. In both crosses, the A1 parent is avirulent (80029 in cross 71 and TV580 in cross 68) and the A2 parent is virulent (88133 in cross 71 and TV618 in cross 68). The open vertical bars represent deletions and the small black horizontal bars represent mutations.

Whereas absence of M5.1 shows a clear correlation with gain of virulence on *R3*, *R10*, and *R11* potato plants, presence of M5.1 in field isolates is much less predictive for the virulence phenotype (Table 2; Fig. 4). Around 50% of the *P. infestans* genotypes that contain M5.1 are still virulent on potato lines with *R3*. In the genotypes that have M5.1, there is still, albeit less pronounced, a correlation between virulence on potato lines carrying the *R3*, *R10*, and *R11* resistance genes. In these cases, the size of the deletion may be smaller, or additional or different mutations either at the locus or elsewhere can result in the acquirement of *R* gene-specific virulence toward more than one *R* gene. Still, the high number of isolates that lack this genomic region (38% of the total number of genotypes and 53% of the isolates virulent on *R11*) suggests that this deletion is associated with an important mutation in Dutch field isolates to gain virulence on potato plants carrying *R3*, *R10*, and *R11*. The importance of the deletion is further strengthened by the fact that this region is also deleted in Mexican and Peruvian isolates.

*R* gene-specific virulence of *P. infestans* field isolates has been the subject of many studies (Andriveau 1994; Drenth et al. 1994; Mooi 1970; Schöber 1972; Shattock et al. 1977). Even in the old clonal lineage, new races frequently appeared that could infect cultivars with new *R* genes (Mooi 1970). Stepwise increases in the number of *R* genes used in commercial potato cultivars were postulated to lead to stepwise increases in the complexity of races in the *P. infestans* population. However, *R* gene-specific virulence in *P. infestans* populations to *R* genes to which it was not exposed has been reported (Andriveau 1994; Drenth et al. 1994). Also, selection pressure on the Dutch *P. infestans* populations to adapt to the new *R* genes has been very limited. The most popular potato cultivars in The Netherlands contain no known *R* genes and some less important cultivars contain mostly *R1*, *R3*, or *R10*, either alone or in combination.

How then can we explain the presence of virulent isolates in these populations? In situations in which *Avr* genes do not contribute much to the fitness of *P. infestans* on potato lines not carrying the corresponding *R* gene, spontaneous mutations can easily get fixed in the population or population frequencies caused by selection in the past might not alter in a new situation. Alternatively, some mutations may effect virulence toward more than one *R* gene. A deletion overspanning several linked *Avr* genes or a mutation in a regulatory gene that acts dominant on several *Avr* genes might have such an effect and could explain our findings with respect to *Avr3*, *Avr10*, and *Avr11*. Virulence toward potato plants carrying *R11*, an *R* gene that has not been used in commercial cultivars, could have hitchhiked along with the selection for virulence toward potato cultivars containing *R3* and *R10*.

Particularly interesting is the finding that in *P. infestans* isolates 80029 and TV580 the chromosomal region containing the *Avr* gene cluster is present in a hemizygous state. Based on hybridization intensities, this is likely to be true for many more isolates, but for definite proof of hemizygosity, sexual offspring must be analyzed. Another hemizygous region in *P. infestans* was found at the mating-type locus (Judelson 1996b). Moreover, the high density of markers in coupling phase found on other linkages groups suggests that hemizygosity also occurs at other loci in the *P. infestans* genome (T. van der Lee and F. Govers, unpublished data). Hemizygous

regions increase variability and may account for spontaneous loss of particular phenotypic characters. For example, Al Kerb and associates (1995) described a sudden change in virulence from race 1.4 to 1.3.4.7.11 in one of their parental *P. infestans* lines upon storage in liquid nitrogen. This may be the result of a spontaneous deletion of a region carrying the relevant *Avr* genes. In addition, in isolates of the clonal lineage US-1, deletions at the M5.1 locus were found, thus supporting the hypothesis that this region is unstable. The telomeric position, the hemizygous state, and the high number of repeats might result in a higher mutation rate for genes located in this region, as was found for *Pi-ta* from the rice blast fungus *Magnaporthe grisea* (Orbach et al. 2000). We are in the process of cloning the *Avr3-Avr10-Avr11* cluster, enabling us to test whether virulence results from loss of *Avr* genes as has been reported, e.g., for *Avr9* from *Cladosporium fulvum* (Van Kan et al. 1991) and *Pi-ta* from *M. grisea* (Orbach et al. 2000).

## MATERIALS AND METHODS

### Cloning of AFLP markers.

The AFLP markers were generated as previously described (van der Lee et al. 1997, 2001) and were separated on 6% denaturing polyacrylamide gels. The gels were dried on gel blotting paper GB002 (Schleicher & Schuell, Dassel, Germany) and labeled fragments were visualized on an X-ray film. Markers were cut from the gel. Accuracy was verified by reexposure of a film to the gel. The tiny pieces of acrylamide were washed twice with 500  $\mu$ l of 10 mM Tris-HCl, 1 mM EDTA for 20 min. Subsequently, the acrylamide pieces were crushed and kept for at least 48 h in 100  $\mu$ l of 1 mM Tris-HCl, 0.1 mM EDTA to allow the DNA to elute from the acrylamide. Five microliters of the eluted DNA was used to reamplify the fragment in 30 PCR cycles (30 s at 94°C, 30 s at 56°C, and 1 min at 72°C) using AFLP primers without the two selective 3' nucleotides. The amplified fragments were run on 1% agarose gels and the fragments were purified from agarose, ligated in pGEM-T-easy vector (Promega Benelux, Leiden, The Netherlands), and transformed to *Escherichia coli* strain JM109 according to the instructions of the suppliers.

### Southern blot hybridizations.

The cloned AFLP markers were used as probes for genomic Southern blot hybridizations. Plasmid DNA was isolated from *E. coli* and digested with *EcoRI*. By agarose gel electrophoresis, the insert was separated from the vector, gel-purified, and labeled with  $^{32}$ P- $\alpha$ -dATP by random prime labeling. Genomic DNA of *P. infestans* was isolated as described previously (Drenth and Govers 1993). From each isolate, 5 to 10  $\mu$ g of DNA was digested with *EcoRI* or *BamHI*, separated on a 0.8% agarose gel, blotted onto Hybond N, and hybridized overnight in modified Church buffer (Church 1984) at 55 or 65°C, followed by three 20-min washes at the same temperature in, subsequently: 0.3 M NaCl, 0.03 M sodium citrate, pH 7.0 (2 $\times$  SSC)/0.1% SDS (twice) and 0.5 $\times$  SSC/0.1% SDS (once). Autoradiographs of the blots were made by exposure of KODAK-Xomat AR film (Eastman Kodak Company, Rochester, NY, U. S. A.) at -70°C with an intensifying screen. Hybridizing fragments were scored visually. The ethidium bromide-stained gels and hybridization patterns obtained with the DNA fingerprint probe RG57 (Goodwin et al. 1992) and

with the single-copy probe *calA* encoding calmodulin (Pieterse et al. 1993) were used as loading controls.

### Characterization of *P. infestans* field isolates.

RG57 DNA fingerprinting patterns, mating type, and virulence phenotypes were compiled from databases from our laboratory and were determined according to methods described by Drenth and associates (1994). Data on the virulence phenotypes of the old US-1 isolates have not been included because they are incomplete. Attempts to repeat the virulence tests and to obtain complete and reliable data were unsuccessful. We, and others, found that determining virulence phenotypes of *P. infestans* isolates that have been in storage for a long time is difficult and unreliable. Over time the isolates tend to lose pathogenicity.

### Data handling and computation.

RG57 fingerprint patterns were used to identify unique genotypes. Isolates with the same RG57 fingerprint were grouped, and only one isolate of each RG57 genotype was used for further analysis. If isolates with identical RG57 genotypes had different virulence scorings on a particular *R* gene differential, the virulence on this particular *R* differential was rated unknown. Statistical analyses were performed using Microsoft Excel.

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## NOTE ADDED IN PROOF

More precise estimations of the length of the M5.1 hybridizing *EcoRI* fragments in the genomic DNA of the avirulent strains revealed that both fragments are slightly larger. What we have indicated here as 'the 800-bp fragment' is more in the range of 1,000 bp, whereas 'the 1,200-bp fragment' is approximately 1,500 bp.

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## CHAPTER 6

# HIGH DENSITY GENETIC LINKAGE MAPS OF *PHYTOPHTHORA* *INFESTANS* REVEAL TRISOMIC PROGENY AND CHROMOSOMAL REARRANGEMENTS

Submitted for publication



## High Density Genetic Linkage Maps of *Phytophthora infestans* reveal Trisomic Progeny and Chromosomal Rearrangements

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Running title: Genetic Linkage Maps of *P. infestans* Key words: Oomycete, Late Blight, Plant Pathogen, Trisomy, Translocation

### ABSTRACT

Detailed analysis on the inheritance of molecular markers was performed in the oomycete plant pathogen *Phytophthora infestans*. Linkage analysis in the sexual progeny of two Dutch field isolates (cross 71) resulted in a high-density map containing 508 markers on 13 major and 10 minor linkage groups. The map showed strong clustering of markers, particularly of markers originating from one parent, and dissimilarity between the parental isolates on linkage group III in the vicinity of the mating type locus, indicating a chromosomal translocation. A second genetic map, constructed by linkage analysis in a sexual progeny of two Mexican isolates (cross 68), contained 369 markers and is thus less dense than the cross 71 map. For some linkage groups the two independent linkage maps could be aligned, but sometimes markers appeared to be in a different order, or not linked at all, indicating the presence of chromosomal rearrangements between genotypes. Graphical genotyping showed that some progeny contained three copies of a homologous chromosome. This trisomy was found for several chromosomes in both crosses. Together, these analyses suggest a genome with a high degree of flexibility which may have implications for evolution of new races and resistance development to crop protection agents.

*PHYTOPHTHORA infestans* (Mont.) de Bary causes late blight, a highly infectious plant disease that is particularly notorious on potato. It brought about the great famine in Ireland in the eighteen-forties and even now potato growers fear late blight. Under conditions favourable for the pathogen, complete defoliation of potato may occur in just a few weeks. Late blight has also filled breeders with despair because R gene based resistance is often lost even before its introduction in commercial cultivars. *P. infestans* belongs to the oomycetes, organisms that despite their fungal-like growth and appearance are unrelated to true fungi. The somatic hyphae are coenocytic and the nuclei are diploid. Meiosis occurs just before mating in well-differentiated oogonia and antheridia. *P. infestans* is heterothallic with two mating types called A1 and A2. Since *P. infestans* does not display visual markers useful for genetic studies, molecular markers are needed for studies on inheritance. Current genetic studies are aimed at elucidating inheritance and cloning of mating type genes (JUDELSON 1996), and genes responsible for fungicide resistance (JUDELSON and ROBERTS 1999) and race-specific avirulence (CARTER *et al.* 1999; VAN DER LEE *et al.* 2001a). Generally the inheritance of molecular markers in crosses appears to be Mendelian (VAN DER LEE *et al.* 1997). However, in some cases deviations were found due to the presence of one or three alleles of a locus in the progeny suggesting the occurrence of translocations, aneuploidy and hemizygous regions (JUDELSON 1996; JUDELSON and ROBERTS 1998; CARTER *et al.* 1999; VAN DER LEE *et al.* 2001b).

To gain better insight in the genetics of *P. infestans* we performed linkage analysis of markers in the F1 progeny of two Dutch field-isolates. Previously we constructed a genetic linkage map (VAN DER LEE *et al.* 1997) and were able to position six race-specific avirulence genes on this map (VAN DER LEE *et al.* 2001a). Here we report more detailed genetic studies. We extended the linkage analysis in the same cross and analysed segregation in another F1 progeny obtained by crossing two Mexican field isolates that are genetically unrelated to the Dutch field isolates. The maps are comparable; however, several cases were found where markers appear to be in a different order or not linked at all. This is particularly evident on linkage group III that contains the mating type locus. The high density maps also revealed trisomic progeny that originates from abnormalities during meiosis.

## MATERIALS AND METHODS

***P. infestans* mapping populations:** Two F1 populations were used for genetic analysis. One is derived from a cross between two Dutch *P. infestans* field isolates, 80029 (A1 mating type) and 88133 (A2 mating type). From this mapping population, called cross 71 and previously characterized by DRENTH *et al.* (1995) and VAN DER LEE *et al.* (1997), 76 progeny were analyzed. The other F1 population is derived from a cross between two Mexican isolates, 580 (A1 mating type) and 618 (A2 mating type). This cross, called cross 68, was generated by L.J. Spielman at Cornell University (Ithaca NY, USA), and first described and characterized by GOODWIN *et al.* (1992). In this study 62 progeny of cross 68 were analyzed. For short periods of time isolates were sub-cultured on rye sucrose medium and for long-term storage isolates were kept in liquid nitrogen.

**Marker generation:** AFLP markers were generated as described by VOS *et al.* (1995), using the restriction enzyme combination of *Eco*R1-*Mse*I with two selective

bases on each side. DNA isolation and template preparation were described before (VAN DER LEE *et al.* 1997). Three types of markers were scored: A markers (genetic model Aa x aa), B markers (genetic model aa x Aa), and H markers (genetic model Aa x Aa). The same person scored all markers visually. Markers were named by the type of marker (A, B or H) followed by the primer combination used to generate the marker and the position in the gel, either as a size estimate (indicated by the letter 's') or as a fragment number (indicated by a 'f'). Markers that segregate in both crosses are called common markers and have an additional prefix C. Markers segregating in cross 71 are shown in upper case (e.g., CAE+AA/M+CAs201.9), while cross 68 markers are shown in lower case (e.g., cae+aa/m+cas 206.5). Scoring accuracy was evaluated by the percentage of dissimilarity in the scorings between replicated individuals over the total number of scored markers using Microsoft Excel. Apart from AFLP markers some RFLP, RAPD and allozyme markers were scored. These markers are also named by the probe, primer or protein used to generate the marker followed by a fragment number or size. Some cross 68 markers were generated by A. Dyer and W.E. Fry from Cornell University (Ithaca, NY, USA) and P.W. Tooley from the USDA (Frederick, MD, USA). For the RFLP, RAPD and allozyme data no quality assessment was performed. Allelic markers originating from the same RFLP or allozyme were treated as separate markers. The mating type was determined by checking for presence of oospores when co-cultured with tester strains of known mating type.

**Linkage analysis:** All individuals generated in the two crosses were analysed for genetic distance using the Dice index within the NTSYS software package (ROHLF, 1993). Isolates with a genetic similarity of 95% or more were considered genetically identical and only one of each set was used in the linkage analysis carried out in JoinMap<sup>®</sup>3.0 (<http://www.plant.wageningen-ur.nl/products/mapping/joinmap.htm>). In the linkage analysis, progeny for which a large number of markers could not be scored reliably, as well as markers that could not be scored reliably on a large set of individuals, were set aside from the data set. Markers were assigned to linkage groups using LOD values ranging from 3.5 to 8.5. Only linkage groups with at least three markers were used for map calculation. To obtain a reliable grouping of the markers, we tested whether (i) the segregation ratio of the markers was in accordance with other markers in the group, (ii) the linkage phase of the markers could be determined relative to the other markers, (iii) the markers were linked to at least two distinct other loci given a LOD value of 1, and (iv) the markers showed clear polarity in the recombination events with other markers. Markers that did not meet these criteria were rejected. Finally we analysed whether the A and B markers separately had enough linkage data to support a single group given a LOD threshold value of 3. To determine the order of markers in the linkage groups we first calculated the order of markers originating either from the A1 parent (Aa x aa), resulting in a so-called A map, or the A2 parent (aa x Aa; the B map). For the larger linkage groups with many A or B markers also A and B maps for each linkage phase (chromosome) were calculated. The marker order was calculated using all linkage data with a LOD score of 1 or higher and a maximum distance of 40 cM with the Kosambi mapping function. A and B maps were used as a fixed framework for the integrated map containing H markers (Aa x Aa) as well as A and B markers. JoinMap<sup>®</sup>3.0 provides the opportunity to set aside markers that cause friction in the map, which is detected by a jump in the  $\theta^2$  value for the map upon addition of each marker.

This  $\theta^2$  was set at the default value 5. When all markers were analysed like this, JoinMap performed a second round attempting to position the markers that initially caused friction and were set aside, thereby using the map calculated in the first round as fixed order and applying the same  $\theta^2$  restriction. Maps were drawn with MapChart (VOORRIPS 2002)

**Alignment of the maps of cross 71 and cross 68:** The maps of cross 71 and cross 68 were aligned using markers that segregate in both crosses, the so-called common markers indicated with a C as prefix. Similar to the integration of the A and the B maps, which was done by markers present in both parents that segregated in the progeny, the alignment was based on markers that had an identical fragment size and an identical intensity, and segregated in both crosses. However, in contrast to markers generated within one cross, markers common in two crosses do not necessarily fit in the same genetic model in each of the crosses. For instance, an A marker in cross 71 (Aa x aa) may be an A (Aa x aa), B (aa x Aa), or H (Aa x Aa) marker in cross 68 and visa versa. The information for the comparison is more reliable when markers of the same type can be compared but more difficult if the comparison involves combinations of marker types. The alignment was done graphically and the position of the common markers was carefully examined using the calculations generated by JoinMap.

**Graphical genotyping of the progeny:** For the identification of trisomic/monosomic progeny we made a graphical display of the markers of each linkage group for each of the progeny. Trisomic/monosomic progeny was detected by the presence or absence of all markers of a particular linkage group regardless of the linkage phase (chromosome) from which the markers originate. This was assayed on linkage groups with five or more markers originating from the same parent with at least two changes of the linkage phase. Graphical genotyping of the progeny was performed in Microsoft Excel and in GGT (VAN BERLOO 1999).

## RESULTS

**Map construction in cross 71:** In cross 71, 223 A markers, 241 B markers and 165 H markers were scored, adding up to a total of 629 markers. Most H markers (Aa x Aa) were scored dominant for presence and absence but some AFLP markers allowed discrimination of the homozygous and heterozygous individuals in the progeny. All markers that passed the quality standard set for this map were AFLP markers. The AFLP markers were scored irrespectively -and in many cases ignorant- of the number of replicates in the progeny. This provided us with a way to estimate the reliability of the data set and in this way we could also cure two pipette and loading errors that occurred during the experimental work. The reproducibility between replicate DNA samples ranged from 95.9 to 100%. Fingerprints derived from DNA isolated from different culture batches of the same individual after retrieval from liquid nitrogen appeared less consistent (95.9% to 99.6%) than fingerprints from DNA isolated from the same batch culture (96% to 100%). Of the 629 markers, 34 were scored on less than 50 progeny and these markers were excluded from further analysis. Similarly, eight progeny had information on less than 520 markers and these progeny were also excluded from further analysis. Linkage analysis was thus performed with 595 markers on 68 progeny. The result of the linkage analysis is shown in Figure 1 and summarized in Table 1. Using a

variable LOD value for grouping (ranging from 3.5 to 8.5) 548 of the 595 markers (92%) appeared to be linked to at least two other markers. For 534 of the 548 linked markers the criteria for reliable grouping (see Materials and Methods) could be met. Markers that did not meet the set criteria were largely H markers for which the linkage phase could not be determined and markers that did not show a clear polarity in the recombination events. In all cases the A- and B-markers separately had enough linkage data to support a single group at a LOD value of 3. In linkage groups containing regions with low marker density (LG IX, LG XI, LG XIII, LG A1-a; Table 1) only a single bridging link at a LOD value of 3 was found but at least two independent links could be established at a LOD value of 1. When the order of the markers in the A and B maps was calculated only a limited number of markers was rejected based on the  $\theta^2$  jump (7 and 4 respectively), indicating that there is not much friction in the data for marker ordering. Also the comparison of linkage maps calculated for markers of a single linkage phase performed on linkage groups I, III, IV, V and VIII, did not reveal differences (not shown). The calculation of the integrated map (A, B, H) using the fixed order from the A and B map appeared to be slightly more difficult as judged by the  $\theta^2$  values and the compression of the genetic distance observed, for instance at the top of LG IV (shown in the left part of Figure 2A). Nevertheless, in most cases the relative position of the H markers in the map with the A and H markers is similar to the map with the B and H markers (as shown for LG IV in Figure 2A). An exception was LG III, which contains the mating type locus. In this case the integration of the A and B map using the H markers caused great difficulties. Apart from the problems encountered in LG III (addressed below), most of the markers could be positioned on the integrated map; 480 after the first and 508 after the second round. The 26 markers that could not be placed with this  $\theta^2$  restriction were not positioned on the map, but are listed in Figure 1 at the bottom of the linkage group to which they are linked. Most of the rejected markers fit best in dense regions where a single scoring error can lead to high  $\theta^2$  jumps. The added value of these markers is low.

On the map we marked the position of six avirulence genes (*AAvr1*, *BAvr2*, *AAvr3*, *BAvr4*, *AAvr10* and *AAvr11*), the mating type locus (*A-MAT*) and one PCR marker (AS1-LOC) representing the S1 locus linked to the mating type locus (JUDELSON *et al.* 1996). The mapping information available for these loci did not meet the quality criteria set for map construction in this study but their map position is relevant for comparison with other genetic studies performed in *P. infestans*. Therefore these loci were placed manually based on the direct distance to the closest markers. In Figure 1 they are written in italics to indicate the lower confidence level. The lower confidence level is also applicable to one manually placed AFLP marker (CAE+GA/M+CGs172 on LG VIII) that was informative for aligning the maps of two different crosses (see below). Finally, we analysed six RFLP markers, five generated by probe RG57 (ARG57.16, BRG57.18, BRG57.7, BRG57.3, RG57.8; GOODWIN *et al.* 1992) and one by cDNA probe APPI122.2. Also for these markers the mapping information did not meet the quality criteria set for map construction in this study. In Figure 1 we listed these markers below the linkage group to which they are linked.

The integrated linkage map of cross 71 comprises 23 linkage groups (Figure 1) of which 13 contain markers from both parents (A, B and H markers). The latter are referred to as major linkage groups and are labelled with roman numbers. The remaining ten minor linkage groups are comprised of markers from a single parent and are named by

their parental origin A1 or A2 followed by a letter. Except for LG A1-a (81.6 cM) and A2-a (52.8 cM) the minor linkage groups are relatively small (3 to 32 cM). Linkage groups III-a, XII, A1-d and A1-e showed strong aberrations in the segregation ratios (listed in Table 1). LG A1-d and LG A1-e contain only A markers of a single linkage phase and also LG VIII and LG IX contain a large number of markers of only a single linkage phase. The major linkage groups have a high marker density with an average interval of 2.1 cM. However, some linkage groups, such as LG XII, or regions, such as the top of LG XI or the lower region of LG IX, are low in marker density, particularly for markers derived from one of the parents or from a specific linkage phase. Some linkage groups contain clusters of markers in coupling phase of a single parent, so originating from a single chromosome. The length of the linkage groups ranges from 3 to 101 cM. Particularly short for the number of markers it contains is LG VI with 22 markers on 10.7 cM; in this case it is even unclear if the map length in cM extends beyond the experimental noise generated by the integration of the A and the B map.

The integration of the A and B map using the H markers caused difficulties in LG III, the LG containing the mating type locus. Closer examination revealed a cluster of six H markers (underlined in Figure 1) strongly linked to the B markers in LG III but not linked at all to any of the A markers in this linkage group. This is remarkable since in this area A markers are present and should have been linked if the H markers on the III-a map would have been on the same position. This indicates that the A and B map were dissimilar for LG III. Consequently, we did not construct an integrated map of LG III but instead constructed separate A and B maps called III-a and III-b. In the two maps the position of the H markers was calculated by adding each and every H marker separately to avoid interference by other H markers. We analysed whether the six H markers from III-b that had no linkage to the A markers in III-a, were linked to A markers on other linkage groups but we could not identify even weak linkage ( $< \text{LOD } 1$ ). Three other H markers had low LOD values to the A markers. The H marker on top of III-b (CHE+CC/M+CTf16) had a LOD value of 1 with some A markers of III-a, and two H markers at the bottom of III-b (HE+GG/M+CCf13A and HE+CA/M+CCf19) were linked to three A markers that also linked together (AE+AA/M+CTs136.3, CAE+AA/M+CTs192.8, AE+AG/M+AAf19A). Although the LOD values were low, the data supported the fusion of these three A markers with LG III-a. Segregation ratios were similar and the direct distance between the A markers as well as between the H markers matched with the calculated distances and the distance of these markers on the III-b map. Therefore we added these markers to LG III-a despite the fact that the significance for linkage is lower (as indicated by the dotted line in LG III-a in Figure 1).

**Map construction in cross 68:** From cross 68, 62 F1 progeny was available. On this progeny 465 AFLP, 17 RFLP, 31 RAPD and two allozyme markers were scored as well as the mating type. The majority of the markers were AFLP markers and the reliability of this data set, as judged by the scoring of replicated samples, ranged from 99.8 to 99.9%. Fifteen progeny had information on less than 443 markers and these progeny were excluded from further analysis. Similarly some of the 513 DNA markers were scored on less than 32 progeny and these markers were excluded from further analysis, along with a set of markers that segregated with aberrant segregation ratio ( $\theta^2 > 5$ ). Linkage analysis was performed with the remaining 425 markers on 47 progeny. Using variable LOD values ranging from 3.5 to 8.5, 393 markers could be grouped in 24

linkage groups. Eight major linkage groups containing both A and B markers, and 16 minor linkage groups, eight with markers originating from the A1 parent and eight with markers from the A2 parent. The order of the markers was calculated but in some of the linkage groups the relatively low number of H markers and the limited number of progeny made integration of the A and B maps more difficult. As a consequence the map generated from the linkage analysis in cross 68 is more fragmented than the map of cross 71 (data not shown). We focussed our mapping efforts on the linkage groups containing markers that also segregated in cross 71 to be able to compare the maps generated with cross 68 and cross 71.

**Alignment of the maps of cross 71 and cross 68:** For alignment of the maps we used markers segregating in both crosses. These common markers were identified by fingerprinting the four parental lines and a number of their progeny side by side followed by careful inspection of the fingerprints for bands with identical length and intensity. We started the alignment of the two maps by grouping the common markers per linkage group. Many of the common markers that grouped with two or more on one linkage group in cross 71 also grouped on one linkage group in cross 68 (Table 2) but there were clear exceptions, for instance in LG I and LG III. We re-tested the grouping of these markers, and all groupings appeared reliable. The numbering of the linkage groups in cross 68 follows the numbering of the linkage groups in cross 71 as much as possible. As a consequence linkage groups in cross 68 indicated by roman numbers are not necessarily major linkage groups and the A1 and A2 linkage groups are not necessarily minor linkage groups. Subsequently, we tested if linkage groups could be merged based on linkage information obtained from the other cross. For example, the exchange of common markers from LG XIII and A2-a suggested that these two groups comprise one linkage group. However, no such merger could be made despite the fact that enough relevant marker data was available.

The different groupings already suggested that integration of the maps constructed from the analysis of the progeny of cross 71 and cross 68 based on the common markers was not possible. We then compared the order of the markers within the five linkage groups for which four or more common markers were available (i.e., LG I, LG III, LG IV, LG VIII and LG XI). In LG VIII and LG XI, alignment of the maps from cross 68 and cross 71 showed minor differences in the order of the markers that remained within the mapping resolution and also the distances between the markers were similar (Figures 2D and 2E). In the comparison of LG IV (Figure 2A) the order and the distance between the markers seemed quite similar for four markers pairs but not for marker pair CAE+AA/M+CAs201.9-cae+aa/m+cas206.5. In cross 71 marker CAE+AA/M+CAs201.9 was mapped distal from markers HE+CA/M+CCf16 and AE+GG/M+CAs10 and also the position of marker cae+aa/m+cas206.5 in cross 68 was clear from the relative position to markers cae+ac/m+cts228.3 and cae+ct/m+tgf17. In these cases all markers originated from the same parent and therefore provided maximum mapping resolution. Also the fact that marker CAE11M15s201.9 was quite distant from the other common markers in cross 71 whereas cae11m15s 206.5 was close to the common markers in cross 68, made an identical position unlikely.

In cross 71 the A and B map of LG III could not be integrated (Figure 1) but there was no problem in the integration of LG III in cross 68. Of the six markers that distinguished III-a from III-b (HE+GT/M+GCf4, HE+CC/M+CCf10,

HE+AC/M+CTs196.9, CHE+AC/M+CTs194.6, CHE+AA/M+CAs152.5 and HE+AC/M+TTf6), two (CHE+AA/M+CAs152.5 and CHE+AC/M+CTs194.6) had a corresponding marker in cross 68 (che+aa/m+cas161.8 and cbe+ac/m+cts182). Both these markers were positioned on LG III in cross 68 (Figure 2C). Furthermore, marker che11m15s161.8 was linked to both A and B markers in LG III of cross 68 demonstrating that the two parents of cross 68 are not dissimilar in this region. Further comparison revealed that the integrated map of LG III in cross 68 is dissimilar to both the III-a and the III-b map of cross 71. The corresponding markers for markers CBE+AC/M+CCs508.7 and CBE+AG/M+CAs307.6 (cbe+ac/m+ccs588.6 and cae+ag/m+cas284.9, respectively) were not positioned on LG III in cross 68. Instead cae+ag/m+cas284.9 was linked (LOD value of 5.9) to marker PEPI in cross 68 on a LG with no other common markers and marker cbe+ac/m+ccs588.6 was linked (LOD value of 6.5) to RG57/1H on LG I part. Vice versa, marker CBE+GA/M+TGf3 corresponding to marker che+ga/m+tgf11 positioned on LG III in cross 68 was not mapped on LG III in cross 71 but on LG VII. Nevertheless, seven other markers and the mating type locus were linked and their relative positions were similar.

In LG I the differences in the order and the distance of four of the six common markers was within the mapping resolution (Figure 2B). In cross 68 marker cbe+ct/m+tgf13a, corresponding to marker CAE+CT/M+TGf11 in cross 71, was not linked to any of the markers from LG I, but this can be due to the absence of B and H markers in the corresponding part of the map in cross 68. However, marker cae+ct/m+ccf6b was located on LG I but the corresponding marker in cross 71 (CHE+CT/M+CCF17) was mapped on LG X.

**Part of the progeny is trisomic for one or more chromosomes:** When a linkage map is constructed, the inheritance of markers in each individual progeny can be visualized in the order of the map by graphical genotyping. This procedure is generally used to identify errors in marker scoring. Such errors often result in apparent double crossing-over events, but these are unlikely to occur. In this study we used graphical genotyping to identify aberrant progeny. In both cross 68 and cross 71, part of the progeny contained all markers from a particular chromosomal pair from one of the parents. One example of trisomy of LG IV is shown in Figure 3. One of the progeny of cross 71, named D12-17, contained all markers from the A1 parent regardless of whether the markers were in coupling phase (same chromosome) or in repulsion phase (homologous chromosome). In addition, D12-17 received a third chromosome represented by LG IV markers derived from the A2 parent. D12-17 thus received both homologous chromosomes of a specific pair from one parent and one recombinant chromosome from the other parent.

The exact number of trisomic progeny is difficult to give since some linkage groups contained only markers of a single linkage phase or the number of phase transitions was too low. As was mentioned above and illustrated in Figure 4 there was a strong tendency for tightly linked markers to occur in coupling phase. Still, by analysing all linkage groups with five markers originating from the same parent with two or more phase transitions, while allowing missing values in case adjacent markers in the same linkage phase were present, we could obtain an estimate. Using these criteria: nine cross 71 progeny were trisomic based on the A map while three progeny were trisomic based on the B map. When the information on the A and B map was combined ten progeny

(14.7%) were trisomic: two for one chromosome, seven for two chromosomes, and one for five chromosomes. For some linkage groups no trisomy was found and consequently none of the progeny appeared to be triploid. Furthermore, we did not find progeny that was monosomic, as would be detected by the absence of all markers from one of the parents for a particular linkage group. Using the same criteria for cross 68, ten progeny were trisomic based on the B map while one was trisomic based on the A map. In total eleven progeny were trisomic and also one possible monosomic genotype was identified. If multi allelic markers were available, the trisomic isolates always correlated with the identification of three alleles of RFLP or isoenzyme markers. However, the presence of either a single or three RFLP or isoenzyme alleles frequently did not correlate to monosomy or trisomy of the chromosome.

TABLE 1

Linkage group of *P. infestans* and statistics on the map construction

Linkage group	Number of markers				LOD group <sup>2</sup>				Rejected <sup>4</sup>			Number of linkage phases			Segregation ratio <sup>5</sup>		
	current	previous	total	a	b	h	C <sup>1</sup>	A+B+H	A	B	Quality	A+B+H	A	B	A	B	
I	I	57	25	24	8	5	5	48	>3	>3	0	6	1	0	2	2	
II	II (part)	24	11	6	7	1	5.5	19	>3	>3	0	3	1	0	2	2	
III-a	III	20+7 <sup>6</sup>	20	0	+7 <sup>6</sup>	2+2 <sup>6</sup>	>5 <sup>7</sup>	20+7 <sup>6</sup>	>3	>3	0	0	0	0	2	n.a.	****
III-b	III	40	0	26	14	8	5	32	>3	>3	3	0	\	0	2	2	
IV	IV	57	23	21	13	6	5	42	>3	>3	2	7	3	4	2	2	
V	V	42	21	14	7	0	4.5	32	>3	>3	0	3	0	0	2	2	
VI	VI (part)	25	7	14	4	1	3.5	20	>3	>3	3	0	0	0	2	2	***
VII	VII	21	2	4	15	1	4	17	>3	>3	1	0	0	0	2	2	
VIII	VIII	42	27	5	10	4	4	37	>3	>3	2	3	1	0	1	1	
IX	IX	39	5	14	20	3	4.5	37	>3	>3	0	2	0	0	2	1	
X	X + A2-e	32	6	13	13	2	4	30	>3	>3	2	0	0	0	2	2	***
XI	A1-b + A2-b + V (part)	41	18	16	7	5	5	31	>3	>2.7	0	2	1	0	2	2	
XII	II (part)	14	1	10	3	1	4	14	n.a.	>3	0	0	0	0	2	2	*****
XIII	VII (part)	9	4	3	2	2	4	9	>3	>3	0	0	0	0	2	1	
A1-a	A1-a	12	11	0	1	2	5.5	12	n.a.	n.a.	0	0	0	0	2	n.a.	n.a.
A1-b	-	7	2	0	5	0	4	7	>3	n.a.	0	0	0	0	1	n.a.	n.a.
A1-c	-	6	4	0	2	0	6.5	6	>3	n.a.	0	0	0	0	2	n.a.	n.a.
A1-d	-	3	3	0	0	0	5.5	3	>3	n.a.	0	0	0	0	1	n.a.	n.a.
A1-e	II (part)	4	4	0	0	1	4.5	4	>3	n.a.	0	0	0	0	1	n.a.	*****
A1-f	VI (part)	6	6	0	0	0	10	6	>3	n.a.	0	0	0	0	1	n.a.	*****
A2-a	A2-a	21	0	21	0	3	6	20	n.a.	>3	1	0	\	0	n.a.	2	n.a.
A2-b	-	5	0	5	0	1	8.5	5	n.a.	>3	0	0	\	0	n.a.	2	n.a.
A2-c	A2-c	15	0	14	1	1	4	15	n.a.	>3	0	0	\	0	n.a.	2	n.a.
A2-d	A2-d	6	0	5	1	1	6.5	6	n.a.	>3	0	0	\	0	n.a.	2	n.a.
TOTAL		548	200	215	133	50	n.a.	480	n.a.	n.a.	14	26	7	4	n.a.	n.a.	n.a.

For each LG the code in the current map, the code in the previous map (VAN DER LEE *et al.* 1997), the number of markers, the LOD value for grouping of the markers, the number of markers positioned/rejected, the number of linkage phases of the markers, and the maximal deviation from the expected segregation ratio is given. If informative, the markers are split according to their parental origin (A, B or H). See materials and methods for nomenclature.

<sup>1</sup> Number of common markers (C) that also segregate in cross 68

<sup>2</sup> LOD value that groups markers in the LG in the integrated map (A+B+H) and the maps of the A and B markers separately

<sup>3</sup> Number of markers positioned in the first (1) and second (2) round of JoinMap<sup>®</sup>3.0 (further explanation in materials and methods)

<sup>4</sup> Number of rejected markers using a restriction of a  $\theta^2$  jump of 5 in the integrated map (A+B+H) and the maps of the A and B markers separately

<sup>5</sup> Maximum deviation from the expected segregation ratio.

\*\*\*\* P < 0.01, \*\*\*\* P < 0.005, \*\*\*\*\* P < 0.001, \*\*\*\*\* P < 0.0005, \*\*\*\*\* P < 0.0001.

<sup>6</sup> LG III is split and the H markers in LG III-a are also present in LG III-b; to prevent counting these markers twice these markers are not added to the total.

<sup>7</sup> For core region see result section

n.a. = not applicable.

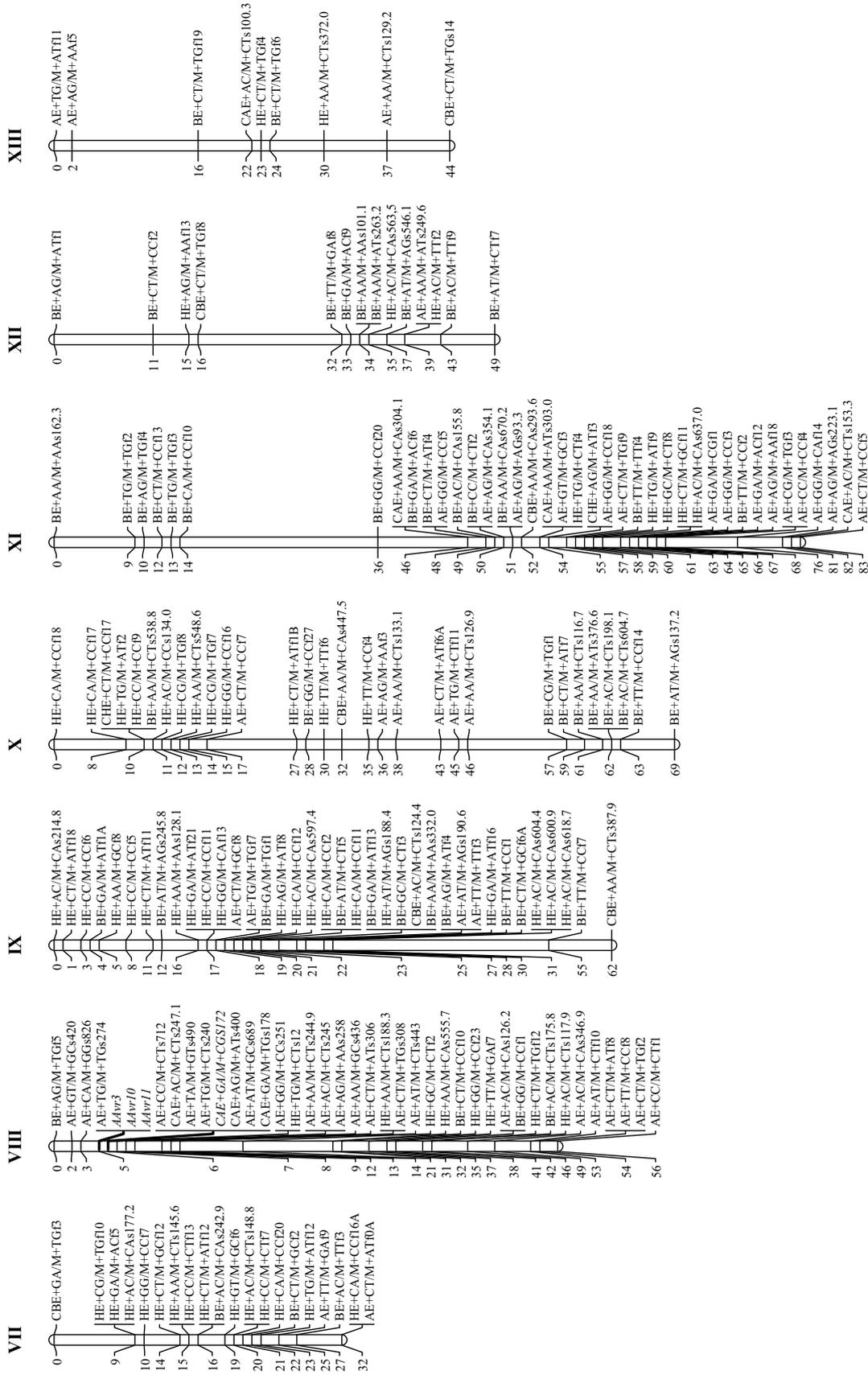
TABLE 2  
Common markers on linkage groups of cross 71 and cross 68<sup>1</sup>

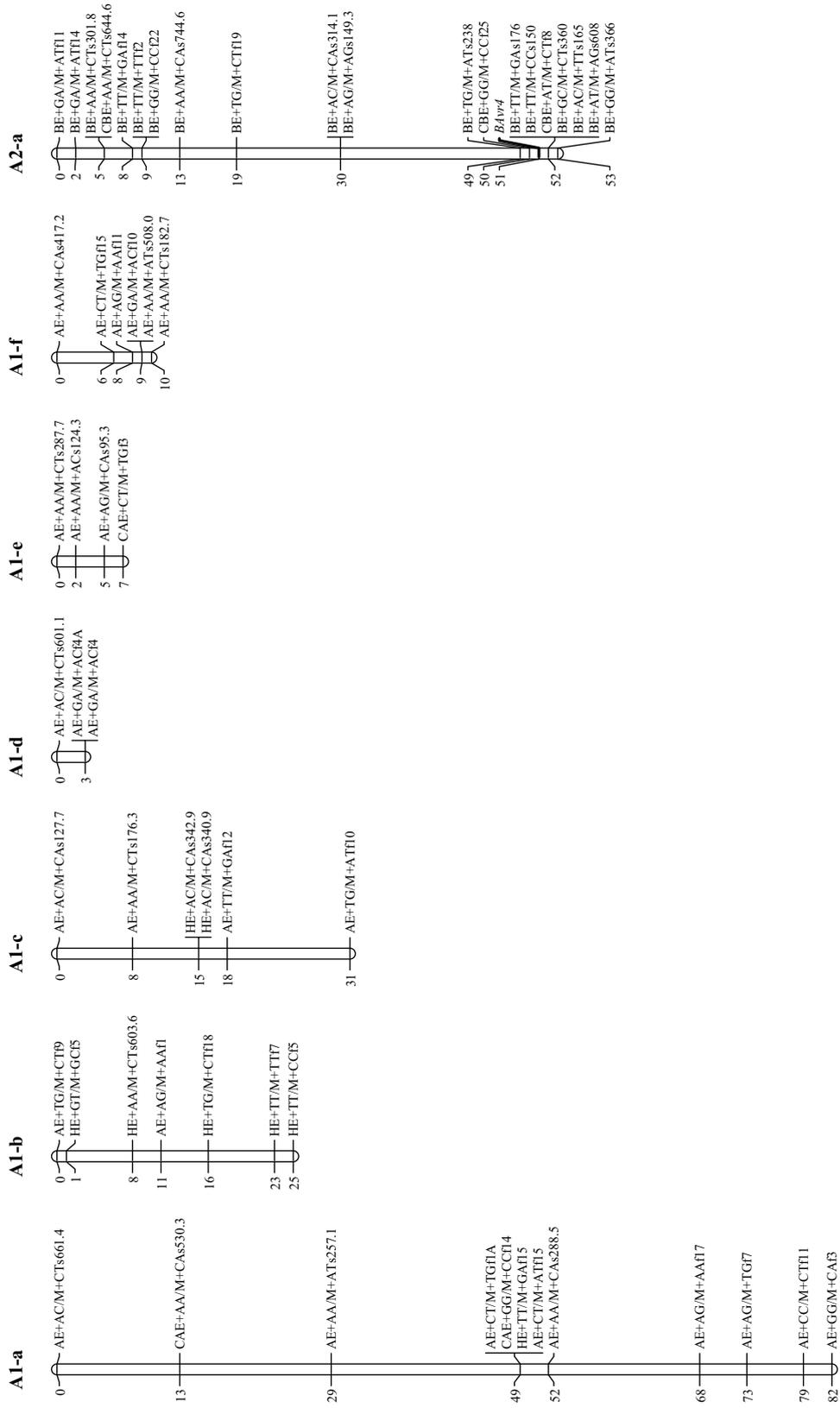
71	68	I	II	III	IV	VI	VIII	IX	X	XI	XIII	A1-e	A2-a	A2-b	A2-c	A2-d	I(part)	68-1	NG <sup>2</sup>
Marker types	A,H	A,B,H	A,B,H	A,B,H	A,B,H	A	A,H	A,B,H	B	A,B,H	A,H	B,H	A,B,H	B,H	A,B,H	A	B,H	A,B,H	
I	4																1		
II		1																	
III			8																
IV				6															1
VI					1														
VII			1																
VIII							4												
IX								2											1
X	1								1										
XI										5									
XII																			1
XIII											1		1						
A1-a																			2
A1-e																			
A2-a						1					1								
A2-b														1					
A2-c															1				
A2-d																1			
NG																		1	3

<sup>1</sup>Linkage groups that do not contain common markers are not listed: LG V, A1-b, A1-c, A1-d, and A1-f of cross 71 and seven linkage groups of cross 68.

<sup>2</sup>NG Not grouped







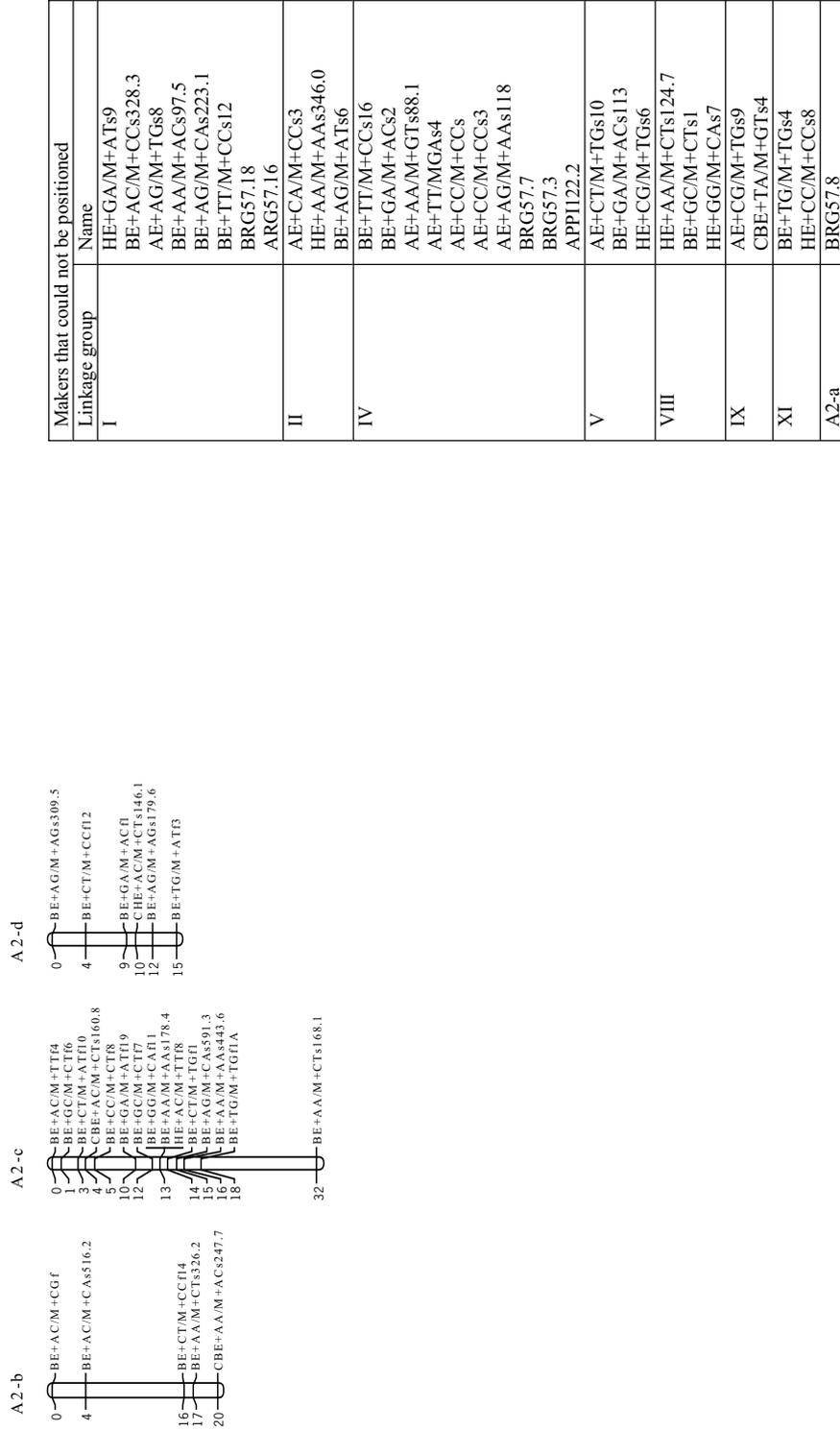
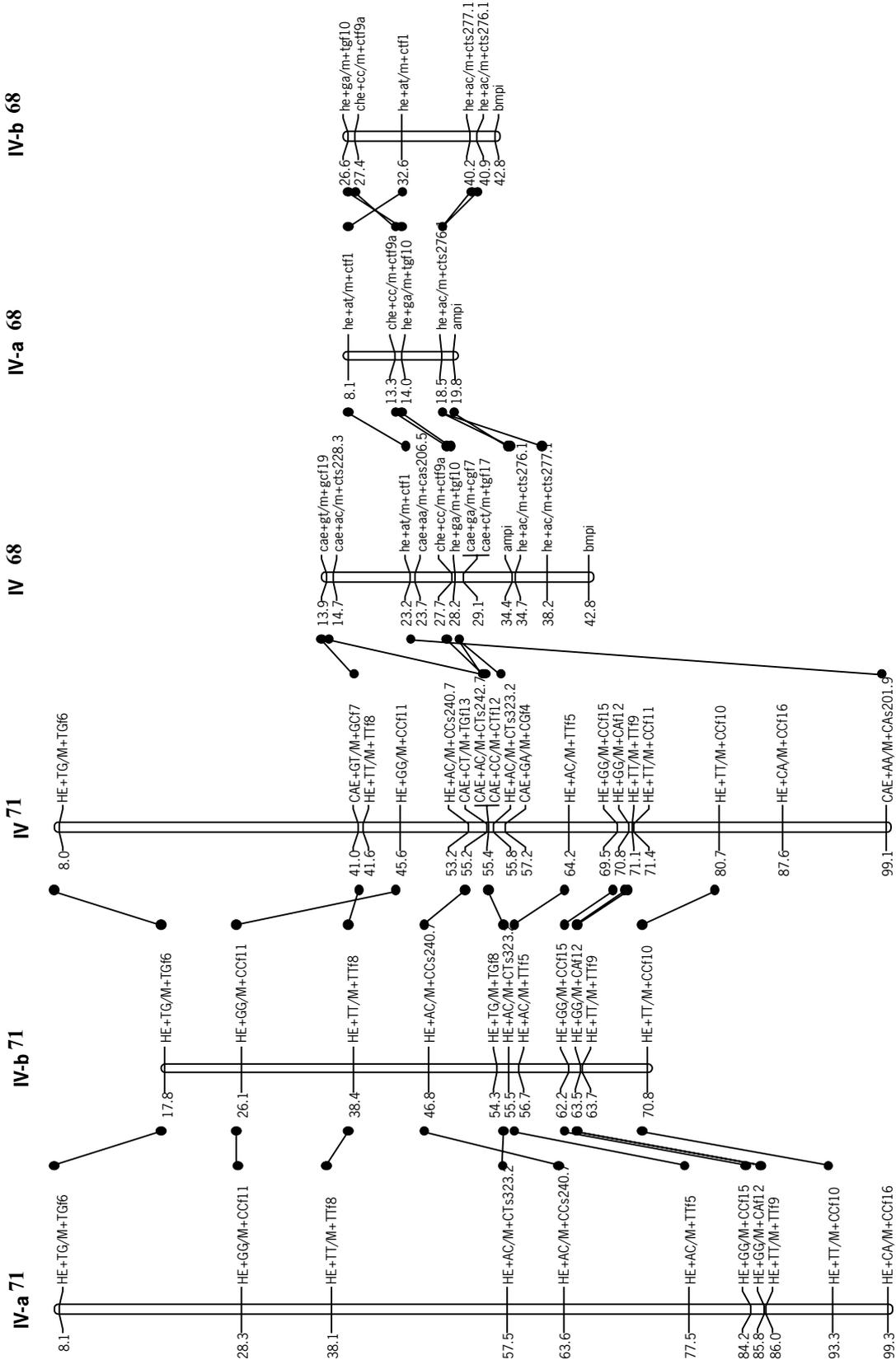


FIGURE 1.-Genetic linkage map of *P. infestans*. The map is based on the segregation of markers in cross 71 and is composed of 13 major linkage groups with A, B and H markers (I to XIII) and eleven minor linkage groups with either A markers (A1-a to A1-f) or B markers (A2-a to A2-d), with or without H markers. Markers are indicated on the right, cumulative distances (in centimorgans) on the left. All markers have prefixes A, B or H according to their origin. Markers with the prefix C, so called common markers, also segregate in cross 68. Nomenclature of the markers is further explained in the text. Markers and loci shown in italics were rejected manually. Markers that were rejected by the  $\theta^2$  jump restriction are listed in the table.

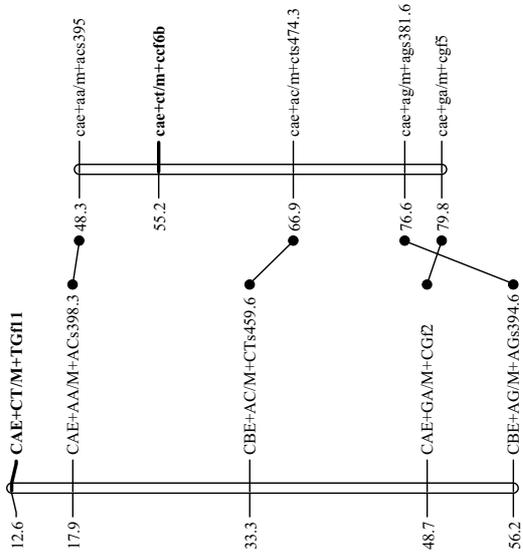
Figure 2-A



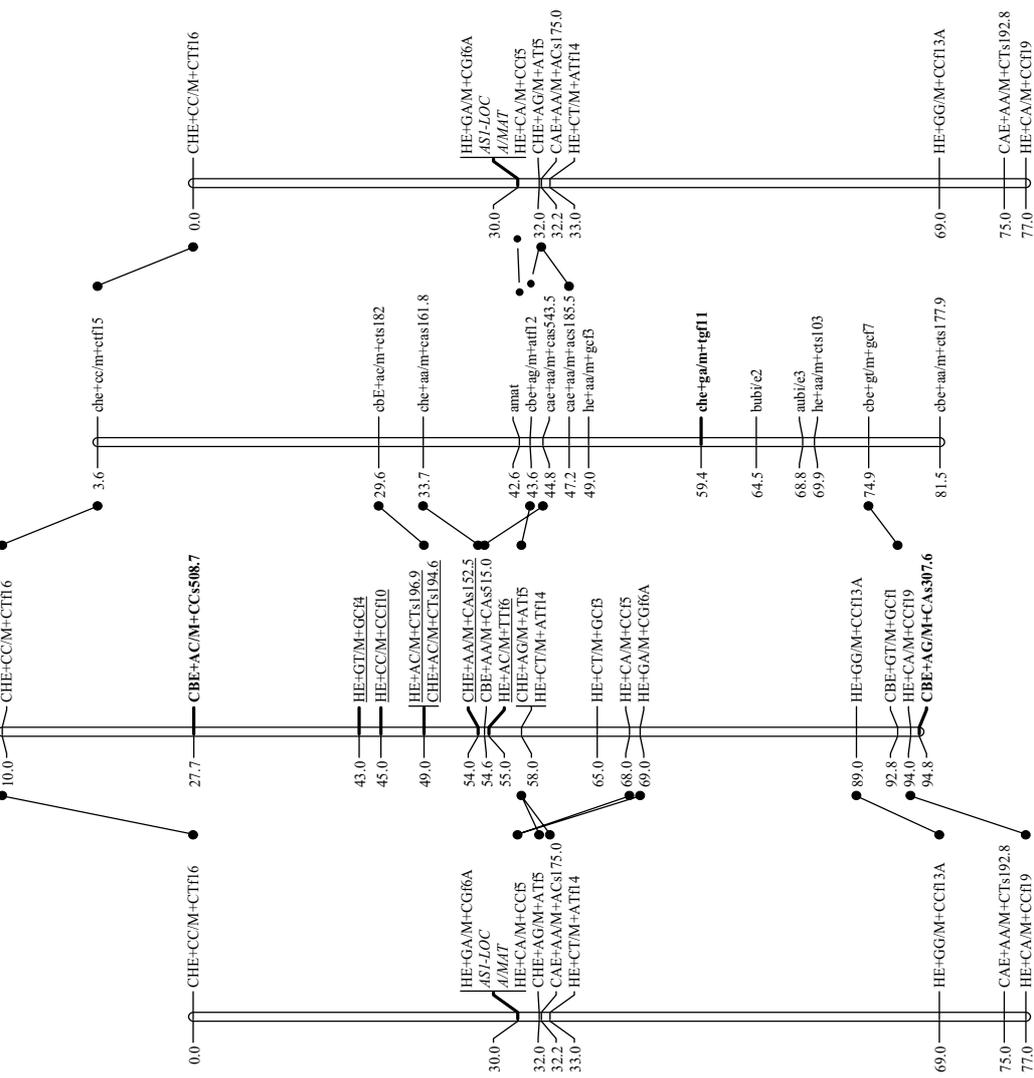
2-B

2-C

I 71



I 68



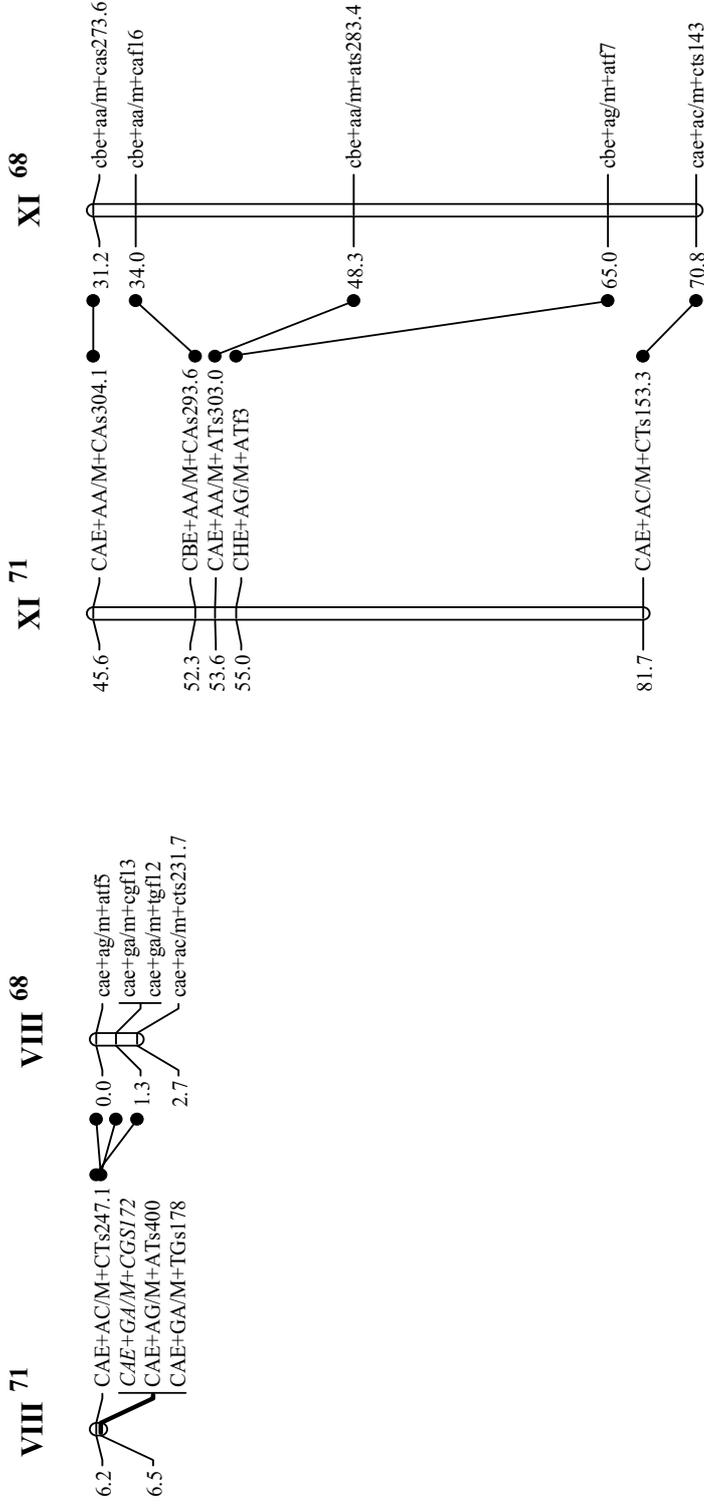


Figure 2.-Alignment of the maps of LG I, III, IV, VIII and XI generated for cross 71 and for cross 68. For clarity only markers also present in adjacent linkage groups are shown and connected by lines. Common markers that should be on the LG are indicated in bold. Note that the marker names of the common markers can be different due to the parental origin and/or the size estimate or fragment number. (A) Alignment of the calculated maps of LG IV, showing the A and B map and the integrated map of cross 71 and cross 68. For each of the two crosses a linkage map was calculated using only the A markers or B markers and the order was fixed. Subsequently, the position of the H markers was calculated in the A and the B maps (IV-a<sup>71</sup>, IV-a<sup>68</sup>, and IV-b<sup>71</sup> and IV-b<sup>68</sup> respectively) and this resulted in a cross specific integrated map (indicated by IV<sup>71</sup> and IV<sup>68</sup>). Note the compression of the H markers in the IV-b<sup>71</sup> map. 2B-Alignment of the calculated maps of LG III-a and III-b from cross 71 with the integrated map of LG III of cross 68. Linkage group III-a<sup>71</sup> is shown twice to facilitate the comparison to both III-b<sup>71</sup> and to the integrated map of LG III of cross 68 (III<sup>68</sup>). 2C, 2D, 2E-Alignment of linkage maps calculated for cross 71 with linkage maps calculated for cross 68 showing parts of linkage groups I, VIII and XI respectively.



Figure 4

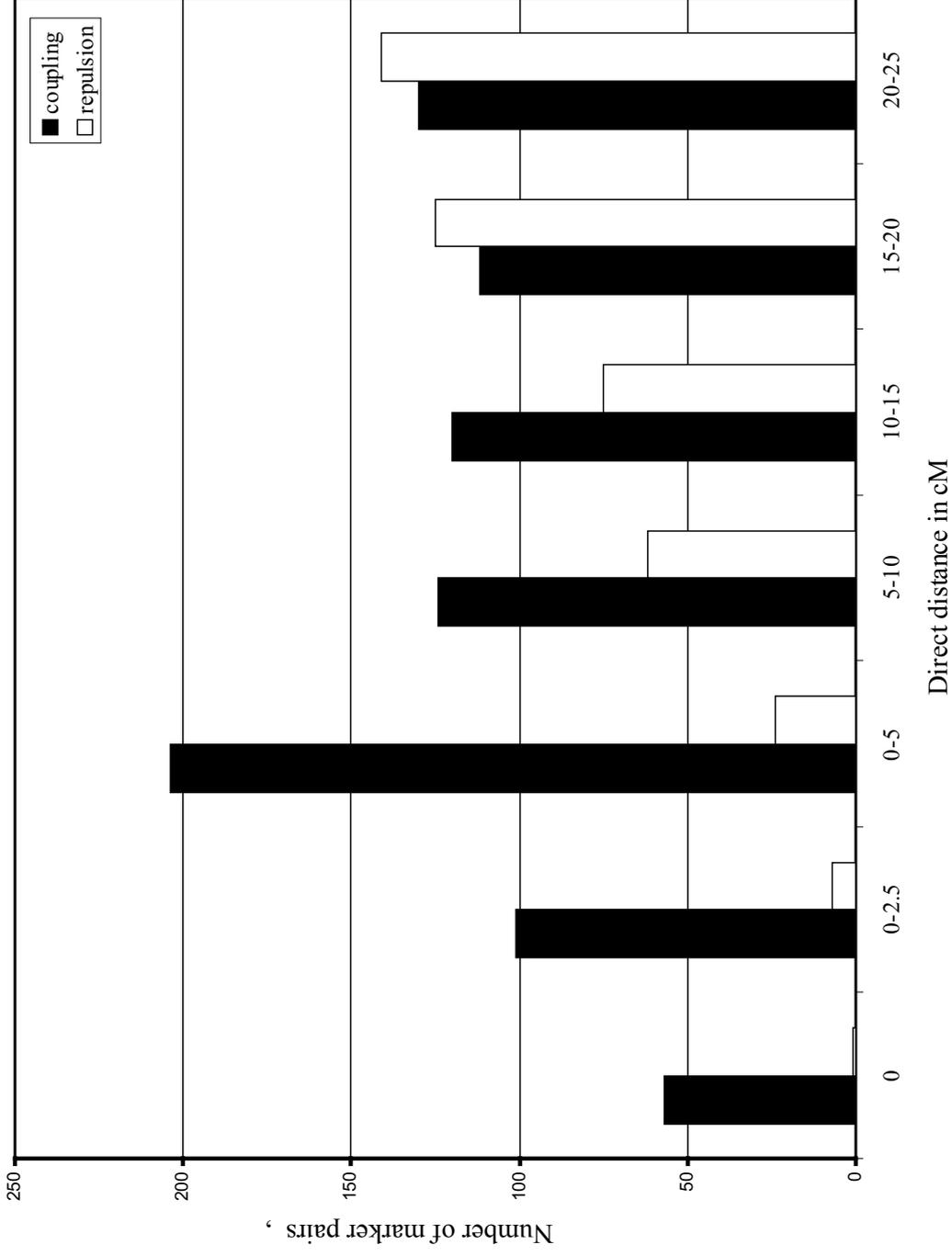


FIGURE 4.-Strongly linked markers are mostly in coupling phase. For 1282 A marker pairs (Aa x aa; Aa x aa) in cross 71 with a LOD value of 3 or higher the linkage phase was determined. Markers are grouped in classes according to their pair wise genetic distance (X-axis) and the number of marker pairs in each class (Y-axis).

## DISCUSSION

Construction of a genetic linkage map is instrumental for inheritance studies in various ways: it generates markers for phenotypic traits, is imperative for positional cloning and it allows detection of aberrations from the Mendelian inheritance. Previous studies in *P. infestans* suggested non-Mendelian inheritance. With the genome wide analysis of *P. infestans* presented here we aim to put these findings in perspective.

**A high density genetic linkage map for *P. infestans* using cross 71:** The markers analysed largely consist of AFLP markers and reliability of this data set as judged by replicate samples of the same isolate was high (nearly 99%). All segregating markers, even those that showed strong deviation of the expected segregation ratios, were scored and analysed. Over 90% of the high quality markers is linked in 23 linkage groups and over 85% of these markers could be positioned. Markers that could not be mapped are largely dominantly scored H markers, which intrinsically are less informative and therefore more difficult to group and position. The robustness of the grouping was tested by building the maps with the two independent marker types, which could be done for all linkage groups except for LG III. The integration of the A and B maps resulted in an integrated high-density map with 508 AFLP markers distributed over 13 major and 10 minor linkage groups. The major linkage groups are dense in markers with an average marker spacing of approximately 2.0 cM. Nevertheless, some regions are low in marker density, particularly for markers originating from one of the parents, or from one linkage phase indicating that the map is far from saturated. Also, the number of linkage groups is remarkably high for the expected 8-10 chromosomes (SAMSONE and BRASIER 1973). It seems, that although more than 90 percent of the markers is linked, some parts of the genome remain uncovered resulting in gaps in the linkage map. This problem may be caused by low polymorphism between the parental isolates for the homologous chromosomes in some regions. The alternative perspective of the situation would be that most markers originate from hyper variable regions. The tight clustering of markers in linkage phase seems to point in this direction. Hemizygous regions were identified before (JUDELSON *et al.* 1996; VAN DER LEE *et al.* 2001b) and may explain this observation.

**Comparison with the previous maps of cross 71:** In general the grouping in the new map fits well with that of the first genetic linkage map of *P. infestans* and is identical to the partial maps presented before (VAN DER LEE *et al.* 1997; 2001a). Compared to the previous map LG I, IV, V, VIII, IX, A1-a, A2-a, A2-c and A2-d remain within the same boundaries upon the addition of the new markers (Table 1). Careful analysis revealed dissimilarity between the III-a and III-b map that was not noticed previously. Some minor linkage groups could be integrated in a major linkage group, which is expected when the number of markers increases. In this way LG A2-e could be integrated into LG X. Similarly A1-b and A2-b and part of LG V were fused to the new LG XI. The most important differences with the previous map are found in LG II, VI and VII, all of which are now split into two or more linkage groups (Table 1). In the case of LG II, markers with distorted segregation ratios erroneously merged three linkage groups in the previous map and LG II is now split in LG II, LG XII and LG A1-e. In LG VI and LG VII problems were caused upon integration of the A and B maps. In the first mapping attempt, the difficulty to construct an integrated map was underestimated and the number of bridging H markers was not high enough for these two linkage groups. LG VI is split in LG VI and LG A1-f, while LG VII is split in LG VII and LG XIII. The current map

was constructed with more markers using more stringent LOD values and stricter criteria. Moreover, the software program JoinMap<sup>®</sup>3.0 is much more sophisticated than the JoinMap1.4 version used for the first map. JoinMap<sup>®</sup>3.0 allows easy identification of markers with aberrant segregation ratios and markers that cause frictions in the map are set aside temporarily. The Windows based user-interface makes it easy to analyse the effect of different settings and of the contribution of individual markers or individual progeny to the map. Nevertheless, construction of integrated maps solely based on dominant markers remains a difficult task. Therefore reassessment of the present grouping will be needed when more markers are added. We anticipate that when more markers are added, groups will merge generating a number of linkage groups that is closer to the expected number of 8 to 10 chromosomes (SAMSONE and BRASIER, 1973). Possibly the analysis of more markers may reveal regions of differences between the two parental isolates as was shown for LG III. With the exception of three markers that were forced to the end of their linkage group by the JoinMap algorithm, the order and distance between the markers in the current map and the previous map is similar, at least within the mapping resolution. Some markers presented in the previous maps did not meet the quality criteria set in the present map mainly based on the number of progeny scored and the polarity of the recombination events. Occasionally markers from different parents “slide” over each other, which is quite understandable given the mapping resolution of the H markers. In cross 68, multi-allelic markers from different parental origin and treated as independent markers, map within 4-10 cM. This indicates that the two parental maps of cross 68 could be integrated well. In cross 71 it is expected that the map integration is even better because more H markers and more progeny were available. Still, integrated maps should be handled with caution since there is no direct linkage information of A to B markers and *visa versa*.

**Translocations and other aberrations at LG III:** In cross 71 the A and the B map of LG III are dissimilar in the region close to the mating type locus. A group of six H markers, in the middle of LG III-b are not linked to markers positioned on LG III-a. This does not involve a deletion, since by nature the H markers (Aa x Aa) are present in both parents. This absence of linkage therefore, is reminiscent of a translocation. We could not identify the repositioning of this region to any of the other linkage groups. The translocation does not seem to correlate to the mating type in *P. infestans* as such, since the parental isolates of cross 68 do not appear to differ in this region. JUDELSON *et al.* (1995) found only two of the expected four possible combinations of gametes in the progeny of some crosses, suggesting balanced lethals, possible generated by balanced translocations. In cross 71 the translocation does not seem to be balanced because all four allelic combinations were found in the progeny. The progeny shows distorted segregation ratios for the A1 and A2 mating type locus and markers from the A1 parent on the same chromosome (VAN DER LEE *et al.* 1997). The progeny mapped in this study was generated from oospores obtained by infecting potato leaves with the parental strains and was recovered from sporulating lesions formed on leaves floating on water mixed with soil containing the oospores (*in vivo*) (DRENTH *et al.* 1995). Remarkably, when progeny of the same parental strains was generated from oospores obtained by co-cultivation on rye medium and germination of these oospores on water agar (*in vitro*), the progeny showed no distorted segregation ratios for the mating type locus nor for markers linked to the mating type (T. van der Lee and F. Govers, unpublished results). The fact that the distorted segregation ratios were found only in the *in vivo* progeny suggests that progeny

with the A1 mating type has an advantage during *in vivo* development, survival and/or growth. In this respect it may be significant that the A1 mating type was distributed all over the world while the A2 mating type was restricted to some areas (FRY *et al.* 1992). Even now, in populations where sexual reproduction occurs, the ratio of A1 to A2 is biased for the A1 mating type, particularly after prolonged periods that favour vegetative growth (ZWANKHUIZEN *et al.* 1998; ZWANKHUIZEN *et al.* 2000). Another remarkable feature is that in all studies reported, including this study, the A1 mating type is dominant, whereas especially A2 isolates of *P. infestans* can be self fertile (SMART *et al.* 2000). It is obvious that the mating type is one of the most challenging and intriguing areas for genetic studies in *P. infestans*.

**Translocations at other linkage groups:** Linkage analysis in cross 68 generated a second genetic map with 369 markers on 24 linkage groups. This map is less dense and more fragmented than the map generated with cross 71. We anticipated that the map of cross 71 could serve as a backbone for the map of cross 68. The integration of the A and B maps based on the common H markers was successful in both, cross 68 and 71, indicating that co-migrating AFLP fragments can be used to integrate genetic maps. We were therefore confident to use the common markers for map integration. We anticipated that the construction of the map of cross 68 would greatly benefit from the information obtained in cross 71 and *visa versa*. Areas with low marker density, caused by low polymorphism between parental isolates of one cross would benefit from markers obtained in the same region in the other cross. Although in general the grouping and order of the common markers is similar in cross 68 and cross 71, we found several cases where it is dissimilar (Table 2, Figure 2). This either means that co-migrating AFLP fragments do not always represent the same fragment and/or that the grouping or order of the markers is different in the parental isolates of cross 68 and cross 71. Given the translocation identified on LG III and the fact that by using the same selection criteria the H markers could be used for map integration but the C markers could not, we hypothesize that translocations occur relatively frequent in *P. infestans*. The use of multi-allelic markers and/or Fluorescent In Situ Hybridisation (FISH) may help to further elucidate this phenomenon. Previously chromosomal translocations were suggested in studies on oomycetes. In *Phytophthora sojae* rearrangements were found within a linkage group (MACGREGOR *et al.* 2002), whereas in *P. infestans* a marker linked to the mating type locus appeared to have translocated to another linkage group (RANDALL *et al.* 2003). Translocations create the possibility to obtain one, two or three copies of a locus or region, which may result in balanced lethals (JUDELSON *et al.* 1995) or in high frequencies of non-viable oospores, as observed in many crosses (KNAPOVA *et al.* 2002). On the other hand, the flexibility to have one, two or three copies of a genomic region can be an advantage in adaptation.

**Trisomic progeny:** Previously, trisomic progeny was postulated based on the presence of three alleles for a locus in *P. infestans* (CARTER *et al.* 1999). However, this was based on single markers and could not be attributed to whole chromosomes. In this study we show that trisomy extends over the whole chromosome. We found significant numbers of trisomic progeny in both crosses (15%) and for specific chromosomes. Yet, we encountered only a single possible monosomic progeny and no progeny that was triploid. Moreover, in cross 68 cases of aberrant segregation of multi allelic markers were found which could not be attributed to trisomy or monosomy. We also identified progeny that was nearly trisomic over the whole linkage as well as a group of isolates that had half

the intensity for a group of markers linked to *Avr3-Avr10-Avr11* (data not shown). In this case, trisomy may have been followed by deletions or by mitotic gene conversion as was found in *P. sojae* (CHAMNANPUNT *et al.* 2001). The parental isolates of cross 71 are both field isolates and their genetic diversity is in line with the diversity found in the Dutch field population. No crossing barriers appear to exist between the parental isolates, as the progeny was no more heterozygous than the parental isolates (data not shown). The parental isolates were also used for mixed inoculations in field trails and numerous oospores were found (DRENTH *et al.* 1995). The cross 71 progeny, including the trisomic progeny, is pathogenic on potato. We therefore think that the trisomic progeny is not an artefact, but reflect the natural situation. Oomycetes have a multinucleate coenocytic mycelium and variable chromosome numbers may be less problematic. We hypothesize that trisomy and subsequent instability of chromosomal regions or loss of complete chromosomes, contribute significantly to the notorious genetic flexibility of *P. infestans*.

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## CHAPTER 7

### GENERAL DISCUSSION



WHAT makes one pathogen more fearful than another? What determines the impact of a pathogen on food and feed production? There are numerous plant pathogens, many of these are opportunistic pathogens causing only mild symptoms or infect plants only during vulnerable developmental stages or during adverse growing conditions. While these opportunistic pathogens are often present without being recognised, others are notorious as they have devastating effects on plants during any time of their development. Some pathogens have close relatives that are not pathogenic, others belong to phylogenetic groups that exclusively contain pathogens. Some pathogens are soilborne and can only migrate over short distances, others produce large numbers of wind dispersed spores that can travel over large distances. For some pathogens no sexual cycle is known, limiting genetic recombination within the population, others have a yearly sexual cycle generating millions of novel gene combinations to be selected for fitness and virulence in the next season. Some pathogens have difficulties to survive outside their host, others can survive for many years until their host appears again. In short, *Phytophthora infestans*, combines the worst cases of all the options listed above, making it a serious threat for sustainable potato production worldwide. But even all the characteristics mentioned above, are not sufficient to explain many of the features found in *P. infestans*. To foster our understanding of this devastating pathogen this thesis describes the transmission genetics of *P. infestans* in general and the genetics of avirulence determinants in particular. The latter is of special interest since presently no durable resistance in potato (*Solanum tuberosum*) is known and resistance from related species was often overcome before introduced into commercial cultivars (TURKENSTEEN 1973; ANDRIVON 1994; COLON et al. 1995). In order to evaluate new resistance sources we need to know how *P. infestans* populations adapt to these resistances.

#### GENETIC STUDIES ON *P. INFESTANS*

Previous reports on the genetics of *Phytophthora* species with expressive titles as “The Peronosporales -- a fungal geneticist's nightmare” (SHAW 1983) served as a warning. Also the absence of a comprehensive genetic linkage analysis and the unsuccessful attempts to identify markers linked to (a)virulence indicated the difficulty to study genetics in *P. infestans*. To acquire a good starting position to answer transmission genetic questions we used a cautious approach. We started with a cross between two Dutch field isolates that generated a large number of viable progeny, called cross 71. The progeny of cross 71 was generated partly by simultaneous infection of potato leaves with the two parental strains (DRENTH et al. 1995) and partly by co-culturing the parental strains on Rye agar plates (GOODWIN et al. 1992). The strains were also used in a field experiment (DRENTH et al. 1995). Given the reports on heterokaryon formation (JUDELSON and YANG 1998; VAN WEST et al. 1999; PIPE et al. 2000) and the reports on the instability of isolates for various phenotypic traits (CATEN and JINKS 1968; GRAND PERNOT 1988), we wanted to assure that the parental lines were genetically uniform and analyzed single zoospore cultures of both parental isolates. Zoospores usually contain only a single nucleus and single zoospore cultures are the best way to separate genetically different nuclei if hosted in the same mycelium (VAN WEST et al. 1999). Comparison of the DNA fingerprints of the single zoospore cultures and their parent revealed that the

parents were homokaryons (Chapter two; VAN DER LEE et al. 1997). We carefully tested whether the mapping populations to be used were all true progeny by examining whether individual progeny did not contain fragments absent in both parental strains and if all of the 320 non-segregating polymorphic AFLP markers (genetic model AA x aa or aa x AA) were found in the progeny. In this analysis all cultures derived from a potato field inoculated with the parental lines of cross 71 were discarded as non-progeny. Likewise part of the *in vitro* oospore cultures appeared to be non-hybrid. For the mapping in cross 71 we continued with the *in vivo* progeny generated on individual plants. All these isolates were found to be true progeny.

Initial analysis of segregating markers in cross 71 and the genetic mapping did not show obvious deviations from the laws of Mendel apart from the segregation ratio for the mating type locus and linked markers (Chapter two; VAN DER LEE et al. 1997). The aberrant segregation ratio for the mating type locus was not reported for any other *P. infestans* cross before, but skewed segregation ratios of markers are not uncommon in other species (ALSTON et al. 1999; JURGENSON et al. 2002). With this first set of data we already noticed that in general the markers appeared to be present slightly more frequent in the progeny than expected. The dominant scored AFLP markers used in cross 71 and the resolution of the map did not allow a more precise analysis. A small set of eight Aa x Aa markers that could be scored co-dominantly, did not show any aberrations. However, when more and more markers were added to the map and the markers were analyzed for the different linkage phases using more sophisticated mapping software a different picture emerged (Chapter six). Several remarkable deviations were found that did not fit the basic rules of Mendel. The first was the identification of progeny carrying more than two copies of a homologous chromosome, e.g., trisomic progeny. The second was the clustering of markers in the same linkage phase. The third was the identification of linkage groups (LGs) that contained only markers of a specific linkage phase, possibly related the incomplete integration of the two parental maps. Finally, mapping problems associated with LG III could be attributed to a translocation between the parental isolates of cross 71. Linkage analysis in a second cross generated from two Mexican isolates did not reveal additional deviations but confirmed that the trisomy, clustering of markers in linkage phase and the hemizygosity found in cross 71 are not specific to this cross but are more general phenomena observed in *P. infestans* (Chapter five and Chapter six; VAN DER LEE et al. 2001a).

#### THE GENETICS OF AVIRULENCE

Since the discovery that pathogens can carry dominant factors that reveal their presence to their host resulting in the activation of host defense responses, there has been a search for avirulence determinants in pathogens to explain cultivar-specific resistance (KEEN 1990). Over the last decades the so called gene-for-gene model gained support as a general mechanism for cultivar-specific resistance or race-specific (a)virulence (DE WIT 2002). Dominant avirulent determinants were identified in many pathogens including viruses (MALCUIT et al. 2000; FELLERS et al. 2002), bacteria (RONALD et al. 1992; WU et al. 2003), fungi (VAN KAN et al. 1991; ROHE et al. 1995) and oomycetes (TYLER 2002). Meanwhile in plants, matching resistance genes were isolated ( JONES et al. 1994; DIXON

et al. 1996) along with a large number of resistance genes that provide cultivar-specific resistance but for which no pathogen trigger has been described yet (DANGL and JONES 2001; MCDOWELL and WOFFENDEN 2003). Also from *Solanum* species resistance genes for *P. infestans* were cloned (BALLVORA et al. 2002; VAN DER VOSSEN et al. 2002). These R genes can be grouped in a few (super)-families according to presence of conserved domains (MCDOWELL and WOFFENDEN 2003). To date there are only a few examples of plant genes that provide effective resistance to pathogens and do not fit this R gene-recognition model. In these cases effective resistance depend on genes encoding proteins that detoxify toxins from the pathogen, (DURMUKH and BRIGGS 1992; HATTA et al. 2002), genes responsible for the accumulation of effective preformed anti-microbial plant compounds (OSBOURN et al. 2003), genes that may encode or mask targets or triggers needed for effective colonization of the plant by the pathogen (RUBIALES et al. 2001) or genes that may be involved in constitutive suppression of host defence response (KIM et al. 2002). Although studies on *P. infestans* by SPIELMAN et al. (1989), suggested that in some cases cultivar-specific virulence in *P. infestans* is dominant, our working hypothesis was that in *P. infestans* cultivar-specific virulence is governed by avirulence genes. This was supported by more elaborate analysis of segregation ratios in different crosses (AL KHERB et al. 1995). Because *P. infestans* is diploid, we could show by transmission genetics studies with linked markers that avirulence is dominant for all six segregating genes in cross 71 (Chapter three; VAN DER LEE et al. 2001A). The fact that avirulence is dominant fits with the gene-for-gene model and postulates a role for recognition for the resistance in the *P. infestans*-potato pathosystem. *Avr1* maps on the distal part of LG IV. *Avr2* is poorly mapped in the middle of LG V; all putative recombinants are avirulent suggesting the presence of a second R gene in the R2 differential potato line matching an additional segregating avirulence gene in cross 71. *Avr3* clusters with *Avr10* and *Avr11* on the distal part of LG VIII. Likewise, *Avr4* was mapped distally on LG A2-a. Given the high density of the map the distal position of the *Avr* genes indicate that they may be located near the telomer. Telomeric position of avirulence genes was also found for several avirulence genes of *Magnaporthe grisea* (DIOH et al. 2000; ORBACH et al. 2000).

As described in chapter four (WHISSON et al. 2001) the markers that are linked to the avirulence genes were used to create physical contigs of the regions in a BAC library of isolate T30-4. Isolate T30-4 is one of the progeny of cross 71 that contains all the segregating cultivar-specific avirulence genes but is still strongly virulent on cultivar Bintje. Several steps were set towards map based cloning of the avirulence genes. However, the lack of genetic recombinants in the regions -partly due to the relative small mapping population- and the difficulty to transform *P. infestans*, a requirement for complementation experiments with candidate sequences, hampered isolation of these *Avr* genes thus far. Another problem is the nomenclature of the avirulence genes. The original differential set of *S. demissum* R genes introgressed in *S. tuberosum* was based on the work of BLACK et al. (1953). Over the years research institutes and breeders developed their own set of differentials ignoring the possibility that a single differential may contain more R genes that may segregate in their offspring. All offspring retained the original R gene number but in fact contain different R genes matching different avirulence factors. Consequently the differential sets of R genes used by the various research groups are not identical nor are the tester strains to validate susceptibility or

resistance. This complicates international collaboration and data exchange. Recent data on the cloning of *R3* gene indicate that already in the original sets of potato differentials of Black and Mastenbroek (BLACK et al. 1953) two different *R3* genes were present (personal communication Francine Govers), which may explain the differences in race typing between our group and our collaborators at the the Scotisch Crop Research Institute (personal communication Steve Whisson). To facilitate future collaborations, there is a need to harmonize the nomenclature of *R* genes and *Avr* genes. In addition it may be practical to rename an *R* gene or *Avr* gene once it is cloned.

#### HEMIZYGOUS REGION

In the search for AFLP markers genetically linked to the *Avr3-Avr10-Avr11* cluster we expected an even distribution of markers in coupling and repulsion phase with the *Avr* genes. Instead, all fifteen tightly linked markers identified in cross 71 were in coupling phase with avirulence (Chapter four; VAN DER LEE et al. 2001b). We hypothesized that a homologous region for this avirulence cluster might be lacking on the other chromosome. Indeed, we identified a region on the distal part of LG VIII that is only present on the chromosome that carries the dominant *Avr* genes. The size of this deletion is yet unknown but preliminary data suggest it is small (Ray Jiang and Francine Govers, unpublished). Unfortunately, the mapping resolution is too low for precise positioning of the avirulence genes relative to this deletion. Furthermore, more elaborate analysis shows that all 42 markers on LG VIII originate from only two linkage phases, one from each parent (Chapter six). This questions whether the aberrant ratio of coupling and repulsion phase markers is caused by a deletion. If it is a deletion the whole chromosome may be absent and the dislinkage measured in cM on LG VIII may not reflect true recombination events but possibly chromosome breakage. Alternatively, for some obscure reason the two homologous chromosomes of LG VIII are invisible on the map. A similar situation can be found on LG IX. Irrespective of the situation on LG VIII and LG IX, (small) hemizygous regions may be frequent and also characteristic for certain regions, as was shown by population studies for the mating type locus (JUDELSON 1996) and the avirulence region of *Avr3-Avr10-Avr11* (Chapter four; VAN DER LEE et al. 2001a). Like trisomy and telomeric positions, hemizygous regions may increase variability. AL KHERB et al. (1995) described a sudden change in virulence from race 1.4 to 1.3.4.7.11 in one of their parental *P. infestans* isolates upon storage in liquid nitrogen. This may be the result of a spontaneous deletion of a region carrying the relevant *Avr* genes. In addition, also in isolates of the clonal lineage US-1 several independent deletions in the hemizygous region close to *Avr3-Avr10-Avr11* were found, again indicating that this region is unstable.

THE CLUSTERING OF *AVR3-AVR10-AVR11* AND ITS IMPACT ON POPULATION GENETICS

Clustering of virulence towards potato lines carrying *R3*, *R10* and *R11* was found in *P. infestans* genotypes despite the large genetic variation and the assumed random mating in the *P. infestans* population in The Netherlands (ZWANKHUIZEN et al. 2000). This might point to a common origin for these virulent isolates. Indeed the clustering of *Avr3*, *Avr10* and *Avr11* on LG VIII suggests a single region determining avirulence towards a number of *R* genes. A strong correlation was found between the absence of marker M5.1 and the virulence of *P. infestans* field isolates on potato lines carrying *R3*, *R10* and *R11* (Chapter four; VAN DER LEE et al. 2001b), suggesting a similar organization and/or regulation of the *Avr3-Avr10-Avr11* cluster in many field isolates. The high number of isolates that lack this genomic region (38% of the total number of genotypes and 53% of the isolates virulent on *R11*) suggests that this deletion is associated with an important mutation in Dutch field isolates towards virulence on potato plants carrying *R3*, *R10* and *R11*. The global importance of this deletion is further illustrated by the fact that this region is also absent in Mexican and Peruvian isolates. Previously, it was postulated that stepwise increases in the number of *R* genes present in potato would result in stepwise increases in the complexity of races in the *P. infestans* population via selection. However, *R* gene-specific virulence in *P. infestans* populations to *R* genes to which they were never exposed was not accommodated for in this model (ANDRIVON 1994; DRENTH et al. 1994). The clustering of avirulence genes suggests that some mutations effect virulence towards more than one *R* gene. A deletion spanning several linked *Avr* genes or a mutation in a regulatory gene that acts dominant on several *Avr* genes might have such an effect, and could explain our findings with respect to *Avr3*, *Avr10* and *Avr11*. Virulence towards potato plants carrying *R11*, a *R* gene that has not been used in commercial cultivars, could have hitch-hiked along with the selection for virulence towards commercial potato cultivars containing *R3* and *R10*. In recent years more reports indicate clustering of (a)virulence towards different *R* genes (KEMA et al. 2000; MAY et al. 2002; DIOH et al. 2000), suggesting that acquisition of virulence towards multiple *R* genes may be more common, with serious implications for the combined use of *R* genes.

## THE IMPORTANCE OF THE CHROMOSOMAL SUB-STRUCTURE FOR INHERITANCE

The nature and the abundance of deviations from Mendelian inheritance in *P. infestans* shows that *P. infestans* is not a model diploid organism. *P. infestans* appears to have a mixture of ploidy levels within a single organism. Whereas diploid organisms such as animals and plants usually have a fixed number of chromosomes with limited levels of heterozygosity between the homologous chromosomes, *P. infestans* has a variable number of copies of various chromosomal regions. While in animals and plants trisomy, translocations and hemizygosity are rare under natural conditions -but can be enforced by chemical treatment or interspecific crosses- these phenomena seem to be more common in *P. infestans*. The gene dosage in *P. infestans* consequently may range between zero due to translocations and/or hemizygous regions, one (due to translocations, hemizygosity and possibly monosomic progeny) two (observed in the regular diploid

state), three (due to trisomy and translocations) and four (combinations of translocation and trisomy or double trisomy). The genome of *P. infestans* seems to be fragmented as the arrangement of sub-chromosomal regions is important for their inheritance. As these deviations from Mendelian inheritance were found in three crosses (Chapter six; CARTER et al. 1999) with viable progeny, these irregularities may also occur under natural circumstances. This is supported by the observed difficulty to determine the ploidy levels of field isolates and the variations found in ploidy levels (TOOLEY and THERRIEN 1987) and may have serious implications for the dynamics of the sexually reproducing *P. infestans* populations currently found in The Netherlands.

#### FUTURE PERSPECTIVES

Oomycetes remain a poorly understood group of organisms and more genetic and genomic research is needed to underpin future control and resistance management strategies. In The Netherlands approximately 50 % of the total volume of crop protection chemicals is used to combat late blight epidemics, signifying the need for more effective control measures. Genomics may reveal new targets and assess the potential of new chemicals currently developed by determining their mode of action. This will result in more effective -possibly curative- chemical control thereby reducing the amounts of chemicals needed. New, more durable resistant cultivars on the other hand, may help to reduce disease pressure, lowering the dependence on chemical control. Still, given the genetic flexibility of oomycetes demonstrated in this study and by others (CHAMNANPUNT et al. 2001; DOBROWOLSKI et al. 2002; JUDELSON et al. 1998), it is wise to be cautious. Multiple simultaneous mutations and/or recombinations may result in competitive adapted genotypes. In the past the genetic variation and flexibility of the pathogen population was largely neglected, inevitably leading to failure in many approaches to control late blight. Evaluation of genetic differences present in a representative set of *P. infestans* isolates and the monitoring of changes in the population will be needed to select the most promising strategies, and to be able to anticipate to population changes. Mapping of traits in combination with a full genome sequence will be instrumental to dissect and identify components of these traits. In addition, the genetic linkage maps developed in this study will assist selection of markers in order to obtain a good coverage of the different genomic regions for population studies. In this respect markers linked to important traits such as (a)virulence may allow more effective evaluation of population changes. Although the challenge to eradicate or even to control *P. infestans* more effectively is enormous, encouragement may be found in the past where for several periods potato could be grown without notable occurrence of *P. infestans* (ANDRIVON 1996; ZWANKHUIZEN and ZADOKS 2002). Apparently the disease is, or at least was, manageable. How these periods came about is not known, what is known is that each time after these silent periods, *P. infestans* returned in a more aggressive form. At the moment it is more difficult than ever to eradicate *P. infestans* because of its world-wide occurrence and the world-wide transport of potatoes and tomatoes. However, increasing knowledge on the pathogen and host resistance should provide opportunities for more effective ways of control in the future.

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## SUMMARY

THIS thesis describes genetic analyses on *Phytophthora infestans*, the causal agent of potato late blight, a highly infectious plant disease. Background information on this pathogen such as its life cycle, its evolutionary position, its threat to potato production and the possible mechanism of cultivar-specific virulence are discussed in chapter one.

Chapter two describes the first genetic linkage map generated for *P. infestans* cross 71. For readers unfamiliar with AFLP DNA fingerprinting this chapter contains a technical description. Its power and reproducibility are demonstrated by the generation of identical fingerprints from asexual single zoospore progeny of the parental isolates used in cross 71. Fingerprinting of the single zoospore progeny also showed that the nuclei of the parents are genetically identical; a prerequisite for further genetic analysis of segregating markers in their sexual progeny. By linkage analysis in the F1 progeny, 183 AFLP markers, 7 RFLP markers and the mating type were positioned on 10 major and 7 minor linkage groups. Our results showed that the mating type is governed by a single locus and that the A1 mating type is dominant. We could find no evidence for balanced lethals for the mating type locus as was reported for some other crosses of *P. infestans*. However, we found segregation ratios of 1:7 for markers linked to the mating type where a ratio of 1:1 was expected. This distorted segregation ratio was only found for markers linked to the A1 mating type.

In chapter 3 we report the mapping of six avirulence genes, *Avr1*, *Avr2*, *Avr3*, *Avr4*, *Avr10* and *Avr11*. AFLP markers linked to these *Avr* genes were selected by bulked segregant analysis (BSA). After screening of over 25,000 markers we found remarkable differences in the number of markers identified for the different *Avr* genes. For *Avr1* we found only a single marker whereas for *Avr4* seven markers were found. *Avr3*, *Avr10* and *Avr11* appeared to be closely linked and for this cluster fifteen markers were found. The number of markers allowed high density mapping for *Avr4* and the *Avr3-Avr10-Avr11* cluster. For *Avr2* no BSA was performed but this gene could be mapped by random markers generated in the mapping project. *Avr1* was mapped on the distal part of linkage group (LG) IV and *Avr2* in the middle of LG V. The *Avr3-Avr10-Avr11* cluster is located on the distal part of LG VIII, while *Avr4* was mapped distally on LG A2-a. The fact that most *Avr* genes map at the end of LGs was noted and its significance has been discussed.

Chapter four describes the construction and screening of a Bacterial Artificial Chromosome (BAC) library of *P. infestans*. A ten genome equivalent BAC library with an average insert size of 98 Kb was generated for *P. infestans* isolate T30-4. T30-4 is one of the progeny of cross 71 that contains all six avirulence genes segregating in this cross, *Avr1*, *Avr2*, *Avr3*, *Avr4*, *Avr10* and *Avr11*. Screening of the BAC library by hybridization with single copy genes and AFLP screening for markers linked to the *Avr* genes showed that the library was representative. With the high-density markers surrounding the *Avr3-Avr10-Avr11* cluster a physical contig was generated and the physical order was compared with the genetic order. In this chapter also the prospects of map based cloning of genes in *P. infestans* have been discussed.

Chapter five describes further efforts to characterize the region of the *Avr3-Avr10-Avr11* cluster. All markers linked to the *Avr3-Avr10-Avr11* cluster were in coupling phase to the avirulence genes, suggesting a deletion. Although later more detailed analysis

showed that this is no exception for this linkage group (Chapter six), we set out to clone the AFLP markers and tested by hybridization whether a chromosomal region was absent. Most markers were not useful for this type of analysis since they contained repetitive sequences. Only three markers hybridized to one or two fragments in Southern blot hybridizations. These results suggest that by BSA we landed twice on the same restriction fragment demonstrating the thoroughness of the BSA. For one of the markers, i.e. M5.1, we showed that no homologue was present in the virulent parent while the avirulent parent contained this marker only hemizygotously. We also showed that the parental isolates of this cross are not unique in this respect as also the avirulent parent of another cross contained this region in the hemizygous state while in the virulent parent this region was absent. The chromosomal deletion as marked by absence of a homolog for M5.1 was also found in 37 percent of a set of 83 genetically different Dutch field isolates. Also in these field isolates this deletion correlates to virulence on potato plants that carry the *R3*, *R10* and *R11* resistance genes. In the discussion it is argued that virulence for *R* genes can be achieved by selection pressure for other *R* genes.

A second generation high-density map for *P. infestans* is presented in Chapter six. Detailed analysis on the inheritance of molecular markers was performed in the progeny of cross 71, a cross between two Dutch field isolates that was used to generate the first genetic linkage map described in Chapter 2. The result was a high-density map containing 508 AFLP markers on 13 major and 10 minor LGs. The map shows strong clustering of markers particularly of those originating from one parent or even from a single parental chromosome. Linkage analysis also showed dissimilarity between the parental isolates on LG III not far from the mating type locus, indicating a chromosomal translocation. A second genetic map was constructed by linkage analysis in a cross of two Mexican isolates. Although this map is less dense and more fragmented (393 markers on 24 LGs groups), for some LGs the maps could be aligned. Generally the order and distance in the two maps were comparable, but in several cases markers appeared to be in a different order, or not linked at all, indicating the presence of chromosomal rearrangements. Additional analysis showed that some of the progeny contained three copies of a homologous chromosome. These trisomic progeny could be found on several chromosomes in both crosses and may be the result of unequal distribution of the chromosomes over the gametes during meiosis.

Finally, in Chapter seven we discuss the findings described in Chapter two to six in relation to the genetic variability and future perspectives of this research for late blight control.

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## SAMENVATTING

Dit proefschrift beschrijft genetisch onderzoek aan *Phytophthora infestans*, de veroorzaker van de aardappelziekte. Deze ziekte leidde tot desastreuze opbrengstverliezen in 1845 met het gevolg dat zich een hongersnood ontwikkelde die ruim één miljoen mensen in West Europa, met name in Ierland, het leven zou kosten. Nog steeds is *P. infestans* één van de meest gevreesde plantenziekten. De jaarlijkse wereldwijde schade wordt geschat op 3 miljard dollar en in Nederland wordt 50% van de chemische gewasbeschermingsmiddelen ingezet om deze ziekte te bestrijden. Omdat *P. infestans* zo'n groot probleem vormt voor de aardappelteelt is de afgelopen decennia veel onderzoek verricht aan *P. infestans* en aan natuurlijke resistentie tegen deze ziekteverwekker in verwante soorten van aardappel. Helaas zijn de tot nu toe gebruikte resistenties alleen effectief gebleken tegen specifieke stammen van *P. infestans*. Stammen die in staat zijn de resistentie te omzeilen zijn virulent en stammen die niet in staat zijn een aardappelplant met het betreffende resistentiegen aan te tasten worden avirulent genoemd. Het gebruik van deze stammen-specifieke resistentie in aardappel kent grote beperkingen. In het veld zijn meestal meerdere *P. infestans* stammen aanwezig, waaronder virulente stammen die zich razendsnel kunnen vermeerderen en zware schade aan het gewas kunnen toebrengen. Hoe *P. infestans* zich onder natuurlijke omstandigheden aanpast aan resistente aardappelrassen is onduidelijk en ook is weinig bekend over de overerving van de genen die verantwoordelijk zijn voor deze stammen-specifieke (a)virulentie in *P. infestans*. Dit is dan ook één van de kernpunten van dit proefschrift. Achtergrondinformatie over de ziekteverwekker zoals de levenscyclus en zijn plaats in de evolutie, de bedreiging die de aardappelziekte vormt voor de aardappelteelt en het mechanisme van stammen-specifieke resistentie in aardappel, wordt beschreven in het eerste hoofdstuk.

Door het DNA van ouders en hun nakomelingen te karakteriseren door middel van zgn. vingerafdrukken en DNA fragmenten die uitsplitsen in het nakomelingschap (merkers) te analyseren is het mogelijk om de overerving van genen en merkers in kaart te brengen. In hoofdstuk twee wordt de ontwikkeling van de eerste genetische koppelingskaart voor *P. infestans* beschreven. Dit hoofdstuk begint met een technische beschrijving van AFLP als methode om een dergelijke vingerafdruk van het DNA van *P. infestans* te maken. Het onderscheidend vermogen en de herhaalbaarheid van deze methode worden geëvalueerd door de identieke DNA vingerafdrukken van klonale vegetatieve nakomelingen die werden gegenereerd van zoösporen afkomstig van twee stammen die gebruikt zijn als ouders voor een kruising. Deze analyse laat ook zien dat de ouders genetisch uniform zijn hetgeen belangrijk is voor het overervingsonderzoek. Als in de nakomelingen DNA merkers vaker dan op grond van toeval verwacht mag worden, samen voorkomen zijn deze merkers aan elkaar gekoppeld. Zo werd een koppelingskaart van *P. infestans* gemaakt bestaande uit 190 DNA merkers verdeeld over tien samengestelde en zeven ouder-specifieke koppelingsgroepen. Verder bleek dat het A1 paringstype dominant is over het A2 paringstype en dat merkers die exclusief gekoppeld zijn met het A1 paringstype veel vaker voorkomen (7:1) dan op grond van toeval verwacht mag worden (1:1).

De koppelingskaart wordt vervolgens in hoofdstuk drie gebruikt om stammen-specifieke avirulentiegenen te positioneren. Om merkers te vinden die nauw met

avirulentie gekoppeld zijn werd DNA van enerzijds, een aantal avirulente nakomelingen en anderzijds, een aantal virulente nakomelingen bij elkaar gevoegd. Vervolgens werden de vingerafdrukken van deze verzamelde DNA monsters met elkaar vergeleken. In totaal werd voor vijf eigenschappen koppeling met ruim 25.000 AFLP fragmenten bekeken. Voor één eigenschap werden geen verzamelde DNA monsters geanalyseerd maar werd alleen gekeken naar koppeling met willekeurige merkers. Op deze wijze werden voor zes avirulentiegenen gekoppelde merkers gevonden waarmee ze geplaatst konden worden op de genetische kaart. Zoals verwacht is avirulentie dominant over virulentie en bovendien bleken de avirulentiegenen *Avr3*, *Avr10* en *Avr11* gegroepeerd in een cluster.

Hoofdstuk vier beschrijft de constructie van een bibliotheek van grote DNA fragmenten van één van de nakomelingen uit de kruising beschreven in hoofdstuk twee. Deze nakomeling is virulent op het ras Bintje, één van de meest gebruikte aardappelrassen in Nederland, maar is niet virulent op aardappelrassen met de resistentiegenen R1, R2, R3, R4, R10 of R11 en zal daarom de zes corresponderende avirulentiegenen bevatten. De grootte van de gekloneerde DNA fragmenten en de representatie van verschillende regio's van het genoom in deze bibliotheek worden beschreven. Tevens wordt er een vergelijking gemaakt tussen de fysische en de genetische afstand in de chromosoomregio waar het *Avr3-Avr10-Avr11* cluster gepositioneerd is. Tenslotte worden de mogelijkheden besproken om deze avirulentiegenen te identificeren uitgaande van hun positie op de koppelingskaart.

Hoofdstuk vijf beschrijft een nadere karakterisering van de chromosoomregio waarop het *Avr3-Avr10-Avr11* cluster ligt. De regio werd geanalyseerd in twee kruisingspopulaties en in een groot aantal veldisolaten uit verschillende jaren en regio's. Deze analyse werd gestart toen, tegen verwachting in, bleek dat 19 nauw gekoppelde merkers zich op hetzelfde chromosoom bevonden als het *Avr3-Avr10-Avr11* avirulentiegen cluster. Deze afwijking kan duiden op een chromosomale deletie op het zusterchromosoom maar dit is met AFLP merkers moeilijk te bepalen. Daarom werden de merkers verder gekarakteriseerd zodat ook analyse van de complementaire regio mogelijk zou worden. Veel merkers bleken gerepeteerde sequenties te bevatten en konden daarom niet omgezet worden in een benodigde unieke merker. Enkele merkers waaronder M5.1 bleken wel uniek. Voor merker M5.1 was echter geen complementair stuk DNA in de virulente ouder te vinden. Verdere analyse liet zien dat de regio alleen aanwezig is in de avirulente ouder en in avirulente nakomelingen. Dit geeft aan dat er in de virulente ouder inderdaad een deletie voorkomt en dat deze deletie ook op één van de twee chromosomen van de avirulente ouder voorkomt. In een groot gedeelte van de veldisolaten (37%) werd ook een deletie in deze chromosoomregio gevonden en er is een duidelijke correlatie tussen de afwezigheid van merker M5.1 en de virulentie op aardappellijnen met de resistentiegenen R3, R10 of R11. De correlatie met virulentie was het hoogst op aardappellijnen met het resistentiegen R11, een gen dat in tegenstelling tot de resistentiegenen R3 en R10, nooit in populaire aardappelrassen werd geïntroduceerd, zodat de *P. infestans* populatie zich daaraan niet kon aanpassen. Mogelijk is virulentie voor R11 in *P. infestans* meegelift met selectie voor virulentie op aardappelrassen met R3 en R10. Dit heeft grote gevolgen voor veredelingsstrategieën die erop gericht zijn duurzame resistentie te verkrijgen door het stapelen of mengen van resistentiegenen.

In hoofdstuk zes wordt de overerving van DNA merkers in twee kruisingspopulaties nader beschreven. Er werden twee koppelingskaarten met een hoge

merkerdichtheid gegenereerd. De eerste koppelingskaart is een uitbreiding en verfijning van de in hoofdstuk twee beschreven koppelingskaart en bestaat uit 508 merkers verdeeld over dertien samengestelde en tien ouder-specifieke koppelingsgroepen. Door het grotere aantal merkers komt de clustering van merkers, met name van dezelfde ouder of hetzelfde chromosoom, veel prominenter naar voren dan bij de eerste kaart. Ook laat de kaart een discrepantie zien tussen de beide ouders in de koppeling van merkers op koppelingsgroep III hetgeen wijst op een translocatie op dit chromosoom. De tweede kaart, gebaseerd op de koppelingsanalyse van een andere kruising, is meer gefragmenteerd. Toch kunnen een aantal koppelingsgroepen van beide kaarten geïntegreerd worden op basis van AFLP merkers met dezelfde mobiliteit en intensiteit. In het algemeen komen de merkers in dezelfde volgorde voor en op vergelijkbare afstanden, maar in een aantal gevallen zijn grote afwijkingen geconstateerd hetgeen wederom wijst op chromosoomtranslocaties. Gedetailleerde analyse van de nakomelingen laat bovendien zien dat een substantieel deel van de nakomelingen trisoom is; zij bevatten drie in plaats van de gebruikelijke twee homologe chromosomen. Deze trisomen zijn kennelijk niet beperkt in vitaliteit omdat ze evengoed in staat zijn aardappelplanten aan te tasten. De frequentie van trisomen in twee onafhankelijke nakomelingschappen wijst erop dat de vorming van trisomen geen uitzonderlijke gebeurtenis is. Waarschijnlijk komen trisomen ook in veldisolaten voor en zijn mogelijk mede bepalend voor de genetische variabiliteit van de *P. infestans* populatie.

Hoofdstuk zeven is een algemene discussie waarin met name wordt ingegaan op de vele afwijkingen van de verwachte overerving die in deze studie zijn gevonden. De gevolgen hiervan voor de genetische variabiliteit van *P. infestans* en de perspectieven voor toekomstige maatregelen ter bescherming van de aardappelteelt tegen de aardappelziekte worden besproken.



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## NAWOORD

Je zult nog wel voor verrassingen komen te staan zei mijn co-promotor toen ik in 1995 begon aan mijn promotie onderzoek. Ze had gelijk, maar in dit boekje worden ook een aantal dingen verklaard en dat is niet alleen mijn verdienste. Veel mensen hebben direct of indirect een belangrijke bijdrage geleverd aan dit onderzoek. Door de hoge omloopsnelheid van medewerkers op de Universiteit en de vele raakvlakken en dwarsverbanden die het onderzoek kenmerkte heb ik met veel mensen samen gewerkt die ik hier wil bedanken.

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Dit boekje beschrijft onderzoek uitgevoerd op het Laboratorium voor Fytopathologie maar er was een leven voor en een leven erna. Beide niet onbelangrijk voor het tot stand komen van dit boekje. Het vorige leven was de start-up tijd bij Keygene. Een boeiende tijd waar ik veel heb geleerd en ik denk met veel plezier terug aan de samenwerking met vele Keygeners. Bij Plant Research International -mijn leven erna- heb ik de analyses gedaan voor het laatste hoofdstuk en de aanmoedigingen gekregen dit proefschrift af te ronden. Hiervoor wil ik met name Gert Kema en Cees Waalwijk bedanken. Ook wil ik het cluster Genetica van Pathogenen van Plant Research International bedanken, een prachtige club waarin je altijd het gevoel hebt dat je in het midden van de wereld staat. Ik ben heel blij dat ik hier de komende tijd mijn bijdrage aan kan blijven leveren.

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Theo

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## *CURRICULUM VITAE*

Theo van der Lee werd geboren te 's-Hertogenbosch op 16 juni 1965. In 1982 behaalde hij zijn HAVO diploma aan het Ds. Pierson college, twee jaar later gevolgd door een VWO-b diploma aan het Sint Jans lyceum. Van 1984 tot 1990 studeerde hij Biologie aan de Katholieke Universiteit Nijmegen (KUN) met stages bij de toenmalige Stichting Voor Plantenveredeling (SVP) in Wageningen, bij de vakgroep planten fysiologie aan de Rijks Universiteit Groningen en bij de vakgroep moleculaire planten fysiologie aan de KUN. In 1990 trad hij als onderzoeker in dienst bij het toen net opgerichte biotechnologisch bedrijf Keygene N.V. waar hij werkte aan de ontwikkeling van merker technologie en de klonering van resistentie genen. In 1995 verruilde hij het onderzoek aan planten voor het onderzoek aan plantpathogenen toen hij als AIO in dienst trad bij het Laboratorium voor Fytopathologie, Wageningen Universiteit. Daar verrichte hij onderzoek aan de genetica van *Phytophthora infestans* en de resultaten daarvan zijn terug te vinden in dit proefschrift. Ook was hij in deze periode actief binnen de onderzoeksschool Experimentele Plant Wetenschappen, onder andere als voorzitter van de AIO-raad. Daarna begon hij in 2000 als onderzoeker bij het toen net opgerichte Plant Research International waar hij als moleculair geneticus onderzoek verricht aan plantpathogenen.

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