Mapping genetic factors controlling potato / cyst nematode interactions

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Mapping genetic factors controlling potato / cyst nematode interactions

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Bibliographic abstract: The thesis describes strategies for genetic mapping of the genomes of the potato cyst nematode and potato. Mapping in cyst nematodes was achieved by AFLP genotyping of single cysts and subsequent segregation analysis in a family of sibling populations. The genetic map of *Globodera rostochiensis* comprises nine linkage groups, a number similar to the haploid chromosome number determined. The low kb/cM ratio suggests that map based cloning of (a)virulence genes may be feasible for this organism.

For potato, a mapping strategy based on catalogued, chromosome-specific AFLP markers facilitated the mapping of the nematode resistance loci *Gpa2* and *Grp1*. *Gpa2* confers specific resistance to *G. pallida* and is tightly linked to a virus resistance on potato chromosome *12*. Locus *Grp1* is identified by QTL mapping. It confers resistance to *G. rostochiensis* pathotype Ro5, partial resistance to *G. pallida* and maps on chromosome *5*. The spectra of nematode resistance loci and their positions on the potato genome suggest that these genes are involved in gene-for-gene interactions.

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Stellingen

1. Variabiliteit in het chromosoomaantal van het aardappelcysteaaltje [Cotten (1959) Nature 183:128; Grisi *et al.* (1995) Fundam. appl. Nematol. 18:67-70] had pas voorgesteld mogen worden indien deze werd aangetroffen in: 1) bivalente configuraties in de metafase van meiose I, 2) meiotisch reduceerde cellen en 3) somatische mitose.
   Dit proefschrift

2. Hoewel de intenties anders doen vermoeden wijzen de gegevens betreffende markersegregatie in de "pseudo-F2 mapping populatie" van *Globodera rostochiensis* op een noodgedwongen monogaam gedrag van mannetjes.
   Dit proefschrift

3. De huidige locus- en alleldefinitie waarin de functie centraal staat [Rieder R., Michaelis A. and Green M. M. (1991) *Glossary of Genetics* 5e ed. Springer Verlag, Berlin, Germany] dient als gevolg van het veelvuldig gebruik van moleculaire markers te worden uitgebreid. Toegevoegd zou kunnen worden dat een locus een chromosomaal segment beslaat dat geflankeerd wordt door "anker sequenties". Allelen van één locus zijn varianten die worden geïdentificeerd met dezelfde anker sequenties.

4. Kwantitatieve resistentie tegen het aardappelcysteaaltje in de aardappel kan beschreven worden middels klassieke gen-om-gen relaties.
   Dit proefschrift

5. Het veelvuldig voorkomen van genen in het plantengenoom die coderen voor eiwitten met "nucleotide binding sites" en "leucine-rich repeat" motieven suggereert een veel bredere rol van deze klasse van genen dan alleen de betrokkenheid bij resistentiemechanismen. De aanduiding "major recognition complexes (MRCs) [Holub, E. B. (1997) Organization of Resistance Genes in *Arabidopsis*. In: *The Gene-for-Gene Relationship in Plant-Parasite Interactions* (Crute I., Holub E. and Burdon J. eds) CAB International, Wallingford, UK] voor de genomische clusters waarin deze sequenties voorkomen lijkt daarom beter op zijn plaats.

6. De hoge mate van homologie tussen het resistentiegen *Rx*, dat de aardappel "extreme resistance" tegen aardappelvirus X verleent en het nematodenresistentiegen *Gpa2* doet vermoeden dat de onderliggende resistentiemechanismen weinig van elkaar verschillen.

7. Afgaand op de hoeveelheid overgedragen erfelijk materiaal per ouder zou een zoon meer op zijn moeder moeten lijken dan op zijn vader.

8. Het grote aanbod van managementcursussen gaat voorbij aan de mensen die moeten leren dit management te ondergaan.

9. Dat de waarheid in het midden zou kunnen liggen is eenvoudigweg niet mogelijk omdat, indien er evenveel argumenten voor twee tegenstrijdige verklaringen zijn aan te voeren, dan beide verklaringen verkozen kunnen worden.

10. Het is valse hoop die (een wetenschapper) doet leven.

11. Het is adembenemend om met zeven kerels de bergen in te trekken.

Stellingen behorend bij het proefschrift getiteld: "Mapping Genetic Factors Controlling Potato / Cyst Nematode Interactions", door Jeroen Rouppe van der Voort.

Wageningen, 15 september 1998

Met mijn opa,
Albert de Rooij
in mijn herinnering

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Introduction

Potato cyst nematodes *Globodera rostochiensis* (Woll.) Skarbilovich and *G. pallida* Stone are tiny roundworms, that spend the better part of their life cycle within the roots of Solanaceous hosts. The nematode starts its life outside the root. After hatching, pre-parasitic second-stage juveniles migrate towards the root of a host plant, penetrate the roots and establish a feeding site near or within the vascular cylinder. This feeding site is called a syncytium and crucial for the further development of the nematode. Once a feeding site is established the motile pre-parasitic nematodes become sedentary and undergo three successive moults before reaching adulthood. The females are globular shaped and appear on the outer surface of the roots where they become visible as white spherical objects. Males become vermiform again, regain their motility and migrate out of the root where they fertilize the females. A remarkable feature of the sex differentiation is that it is determined epigenetically, with males increasing in frequency under conditions of high nematode densities or poor nutrition (Mugniéry and Fayet 1984). After fertilization, hundreds of eggs can develop within the females body. Subsequently, the female dies and her cuticle forms a protective sheath (called a "cyst") for the eggs containing second stage juveniles in diapauze.

Both species of the potato cyst nematode originate from the Andean region of South-America where they co-evolved with their Solanaceous hosts (Evans and Stone 1977). They are thought to be introduced in Europe after 1850 and cause substantial losses in potato crops.

Potato cyst nematodes: a major yield-limiting factor in potato: The history of the potato cyst nematode outside its centre of diversity starts with a report on damage to potato plants in an allotment garden at Rostock (Germany, the name '*rostochiensis*' refers to this place) in 1913. Since then, infestations of potato cyst nematodes were found in various European countries and the USA (summarized in Oostenbrink 1950). Potato cyst nematodes are the causal agent of a disease called in Dutch: "aardappelmoeheid". The disease is characterized by yellowing of the leaves, desiccation and starvation of the plant and perturbation of tuberization. The effect of potato cyst nematode infestations on potato growing is twofold. Firstly, heavily infested plants hardly form tubers. Worldwide tuber yield losses due to potato cyst nematodes infestations are estimated to be 10% annually (Oerke et al. 1994). Secondly, the nematodes are very persistent in the soil. Only the

application of biocides reduces the nematode populations to a reasonable extent. The increased environmental awareness resulted in stringent measures taken by the European community to reduce the use of these chemicals. As an alternative control measure, crop rotation is applied. However, given the fact that potato is a cash-crop, crop rotation is from an economical point of view less desirable. Therefore, future potato farming will presumably rely on the availability of a range of resistant cultivars. These resistant potato cultivars can be grown more frequently and reduce existing nematode populations without the use of chemical methods. The biology of the cyst nematode is fundamentally different from most plant parasitic bacteria and fungi. Especially, the low multiplication rate and the limited mobility of cyst nematodes explain why host plant resistance is durable. Developing tools to trace and finally clone resistance genes in potato and (a)virulence genes in the potato cyst nematode are the subject of this thesis.

Gene-for-gene relationships: The mechanisms underlying plant defense against invading pathogens are incompletely understood. While studying the interaction between flax (*Linum ultissimum*) and the rust fungus *Melampsora lini*, Flor (1956) postulated a gene-for-gene relationship between this plant-pathogen and its host. A gene-for-gene relationship implies that resistance or susceptibility of a host plant not only depends on a genetic factor in the plant but also on a genetic factor in the pathogen. Since then, it has been shown that the outcome of many host-parasite interactions is determined by the combination of a single major resistance (*R*) gene in the host and single major avirulence (*Avr*) gene in the parasite. If no resistance gene is present in a host plant, the parasite will be able to multiply on it, irrespective whether this parasite contains the gene for avirulence or not. On the other hand, if the parasite lacks a specific avirulence gene, the plant pathogen will successfully invade the host, irrespective whether the host plant harbors the corresponding resistance gene or not. Host plant resistance will only be functional if both the resistance gene product and the corresponding avirulence gene product are present. In Table 1, the possible gene combinations and the corresponding disease reaction types are summarized for a diploid pathogen and its diploid host.

Table 1: Features of a gene-for-gene relationship involving two interacting gene pairs. An interaction phenotype scoring "-" represents complete incompatibility (*i.e.* resistance/avirulence) and a score of "+" represents the highest level of compatibility (*i.e.* susceptibility/virulence). An intermediate phenotype ("+/-") is expected when only one of the matching *Avr-R* combinations occurs.

Host\Pathogen genotypes	*Avr1/Avr2*	*avr1/Avr2*	*Avr1/avr2*	*avr1/avr2*
R1R2	-	-	-	+
r1R2	+/-	+/-	+	+
R1r2	+/-	+	+/-	+
r1r2	+	+	+	+

The population genetic consequence of dominant genes for resistance in the host and dominant avirulence genes in the pathogen is that genes for avirulence may disappear as the result of selection pressure by host resistance. Whenever recessive virulence genotypes appear in the pathogen population, the resistance in the host breaks down. Plant breeders may then introduce another gene for resistance in the plant which extends the resistance of the host beyond the range of the newly acquired virulence characteristics of the pathogen. A new variety of a crop remains resistant until another virulence characteristic emerges in the pathogen population.
Gene-for-gene interactions involving a Mendelian analysis on the inheritance of resistance in the host and avirulence in the pathogen have been determined for at least 14 plant pathogen associations. In addition, for at least 34 other combinations GFG relationships have been postulated in which no formal proof can be given due to the lack of a sexual cycle in pathogen reproduction (Thompson and Burdon 1992; Thompson 1994). In the latter case, GFG relationships have been demonstrated through the interaction of known resistance genes with cloned avirulence genes. It is noted that the model on gene-for-gene relationships is an over-simplification. Nevertheless, it is a useful prediction on the interaction between plants and their pathogens.

Gene-for-gene relationships have also been postulated for plant parasitic nematodes and their hosts (Jones et al. 1981). However, only in case of virulence in *G. rostochiensis* and the *H1* resistance gene from *Solanum tuberosum* ssp. *andigena* CPC1673 a formal proof for a GFG relationship has been established (Janssen et al. 1991). As will be discussed in the final chapter of this thesis (**chapter 9**), gene-for-gene relationships are likely to operate in the majority of the potato cyst nematode - potato interactions which are observed nowadays in the field.

Major resistance in potato: From the moment onwards that the seriousness of potato cyst nematodes as a major limiting factor in potato was fully recognized, a search for resistance has been undertaken in wild potato species. Potato has one of the richest genetic resources of any cultivated plant and numerous *Solanum* accessions have been tested for resistance against potato cyst nematodes. Resistance loci have been identified in the species *S. tuberosum* ssp. *andigena* Hawkes CPC1673 (loci *H1* and *Gpa2*; Huijsman 1955; Arntzen 1994), *S. kurtzianum* Bitt. et Wittm. (loci *A*, *B*; Huijsman 1960), *S. multidissectum* Hawkes (locus *H2*; Dunnet 1961), *S. spegazzinii* Bitt (loci *Fa, Fb* (= *Gro1)* and *Gpa*; Ross 1962; Barone et al. 1990; Kreike et al. 1994) and *S. vernei* (*GroV1*; Jacobs et al. 1996).

The above mentioned resistances are characterized by single inheriting genes. This type of resistance differs from more complex inheriting resistance which is based on multiple, unlinked loci. Complex resistances are inherited as a quantitative trait in which the offspring cannot be divided into discrete parental phenotypic classes (*i.e.* resistant or susceptibility) but are described by phenotypes displaying a continuous level of variation. Most broadly operating resistances against potato cyst nematodes are thought to be complex inherited. To provide more insights into this type of interaction, a study on the genetics of complex resistance has been carried out (**chapters** 7 and **9**). Complex inheriting resistance to potato cyst nematodes have been found in *e.g.* various accessions

of *S. vernei* (Goffard and Ross 1954; Ross 1986) and in *S. tuberosum* ssp. *andigena* CPC2802 (Dale and Phillips 1982).

Virulence in potato cyst nematodes: Potato cyst nematode populations respond differently to potato genotypes harboring various kinds of resistance genes. This observation resulted in the development of classification schemes which discriminate potato cyst nematode populations on the basis of their virulence characteristics. The international pathotype scheme recognizes five pathotypes within *G. rostochiensis* and three within *G. pallida* (Kort et al. 1977). After its appearance, this scheme has been seriously criticized (Trudgill 1985; Nijboer and Parlevliet 1990). A pathotype is a general phytopathological concept that presupposes: (1) knowledge on the loci for resistance and avirulence and (2) expression of a particular type of (a)virulence by all individuals of a certain pathotype. In case of the international pathotype scheme for potato cyst nematodes, these criteria are only met for the interaction between *G. rostochiensis* pathotype Ro_1 and the *H1* resistance gene. The inaccuracy of this commonly used pathotype scheme has obstructed the breeding for resistance against this pathogen.

To obtain a better insight in the genetic structure of potato cyst nematode populations, an extensive survey was performed by Folkertsma (1997b). About 300 populations were classified on the basis of various molecular and virulence characteristics. This study revealed that for *G. rostochiensis*, the classification on the basis of molecular characters generally coincides with the classification on the basis of virulence characters. Each group probably represent the descendants of one or a few similar introductions. For *G. pallida* populations only the populations which appeared to be extensively diverged at the molecular level could be discriminated on the basis of specific virulence characteristics. However, a spectrum of virulence and molecular differences were found among the major group of *G. pallida* populations analyzed and it was not possible to unequivocally group these populations on the basis of shared virulence characteristics. Nevertheless, it can be argued that also these populations have a common origin.

Identification and mapping of resistance and avirulence genes: At the start of this thesis, a few potato accessions were available which contain a broad spectrum of resistance. These potato accessions were the result of 40 years of breeding in an attempt to accumulate resistances from wild species into new breeding material (H. Vinke pers. communication). At that time, breeders and farmers were confronted with potato cyst nematode infestations for which no profound idea on the (a)virulence characteristics existed. To increase the resistance spectra of new accessions, various sources of resistance, irrespective of their monogenic or polygenic inheritance were therefore randomly hybridized to produce broadly resistant breeding lines. For these breeding lines, no knowledge is available for the number and specificity of the genes involved in the resistance.

To unravel the complex resistance phenotypes at the one hand and the (a)virulence characteristics of potato cyst nematode populations on the other hand, the construction of genetic maps are indispensable. Genetic linkage maps represent the relative order of genetic loci on the chromosome and provide the ability to determine the contribution of

genomic regions on the inherited trait. Genetic maps could be constructed after the discovery of the basic principles of inheritance by Mendel (1866) and the role of chromosomes therein (Boveri 1902; Sutton 1903). However, it lasted until 1913 that multiple traits could be linearly arranged to represent the order and genetic distance of these traits on the chromosome (Sturtevant 1913). Since then, researchers identified, cataloged and mapped various single gene markers. The first genetic maps for a crop species appeared in the early thirties (*e.g.* MacArthur 1934). The type of genetic loci studied to construct these classical maps were morphological traits. The limited number of loci which segregated in one cross, required a large number of segregating populations for the construction of these maps. In some extensively studied species, tester lines were developed from crosses between highly divergent parents to carry many genetic markers. However, the lack of genetic markers and the difficulties in developing tester lines has hindered the construction of linkage maps in many important species. Moreover, the number of genetic loci which could be studied within one species was never sufficient to cover the genome of an organism.

The advent of molecular (DNA) markers has been a major advance in the construction of genetic maps. DNA markers, like restriction fragment length polymorphisms (RFLPs; Botstein et al. 1980), randomly amplified polymorphic DNA (RAPD; Williams et al. 1990) and AFLPs (Vos et al. 1995) are based on the detection of genetic variation within a defined DNA segment. The techniques in which these DNA polymorphisms are detected differ from each other in practicability, informativity and reproducibility. DNA markers offer the advantage that 1) the number of detectable loci in a suitable cross is nearly unlimited which allows the construction of dense linkage maps, 2) only a single genetic cross needs to be studied 3) in some cases, the codominance of alleles at a given locus is resolved 4) no pleiotropic effects of individual markers are encountered and 5) are insensitive for environmental conditions.

Genetic maps provide the ability to assess the relationship between a phenotype and the underlying genotype by significant association between the occurrence of flanking markers and a phenotypic trait in the progeny. Similarly, complex phenotypes can be resolved into separate genetic components. In addition, the contribution of each separate gene to the trait can be quantified and the type of interaction between the different loci can be determined. Loci contributing to a phenotype are defined by molecular markers flanking the genomic region. The flanking markers can be used as starting points for isolation of the genes involved.

For several plant species detailed (> 500 markers) genetic maps are available, including *e.g. Arabidopsis* (Reiter et al. 1992; Hauge et al. 1993), rice (Harushima et al. 1998), barley (Qi et al. 1998), tomato and potato (Tanksley et al. 1992). The construction of linkage maps for plant pathogens has stayed far behind the developments in crop genetics.

Towards cloning of resistance and avirulence genes: Many disease resistance genes in plants and a few avirulence genes in fungi (Hulbert et al. 1988; T. van der Lee pers. communication) have currently been localized by genetic mapping. However, the mechanisms by which the gene products (inter-)act can only be resolved by isolation of

the genes themselves. When the function of such gene is unknown, independent methods are required to trace the genes underlying a phenotype.

Map based cloning offers the opportunity to isolate any gene in the genome which phenotype can be precisely mapped. The approach is based on the physical linkage of the gene to flanking molecular markers. Once tightly linked markers are identified, one may walk through the genome by overlapping DNA clones to the gene of interest. Map based cloning has been restrained for a long time by the absence of high throughput markers and by feasible methods to clone high molecular weight DNA. However, the cloning of genes based on their map position has greatly been facilitated by two major achievements. Firstly, with the development of the AFLP marker technique (Vos et al. 1995), a high throughput marker technique has become available. AFLP allows the detection of up to 100 DNA fragments per assay from minute amounts of DNA which can all serve as potential markers. Secondly, bacterial artificial chromosomes (BACs; Shizuya et al. 1992), have facilitated the cloning of large fragments of DNA. By use of these techniques a targeted genomic region can be saturated with DNA markers. These markers can then be used to screen a BAC library; the identification of BAC clones and the conversion of BAC ends into new markers should then be feasible to build an overlapping set of BAC clones. Subsequently, candidate genes can be isolated from the implicated genomic region. Evidence that the target gene is cloned is obtained when transformation of the candidate gene into a recessive genotype results in the desired phenotype.

When profound knowledge on the biochemistry of the phenotype exists, a comparison between the wild type and the mutant phenotype may lead to the isolation of a protein which forms then the basis of the isolation of the gene involved. Alternatively, mutants can be induced via *e.g.* insertional mutagenesis. Insertional mutagenesis is mostly carried out by the use of transposable elements. Transposons are mobile DNA segments that migrate through the genome by a process of excision and reinsertion thereby sometimes causing phenotypically visible mutations. As the sequences of the inserted transposon are known, these sequences (the "tag") can be used as a starting point for characterization of the flanking DNA. Transposon tagging is a feasible cloning strategy when one has an easily screenable mutant phenotype (*e.g.* for isolation of the *Cf9* gene from tomato, five out of 37,000 transposon lines displayed the desired mutant phenotype (Jones et al. 1994)). However, for cloning of genes involved in the potato cyst nematode - potato interaction, the resistance assays are too laborious for screening thousands of mutant phenotypes.

Strategies for gene mapping: outline of this thesis: This thesis aims at getting insights in the genetics of the two parties involved in an intimate plant-animal interaction: the potato cyst nematode and its host potato. Chapters 1 and 2 focus on the genetics of the potato cyst nematode, chapter 3 to 8 deal with the (fine) mapping of nematode resistance genes in potato.

For potato cyst nematodes, genetic data were scarce and conflicting. Before a genetic map was constructed, we ensured ourselves on the stability of the chromosome number and proper chromosome segregation in the meiosis. Therefore, a study was made on the cytogenetics of *G. rostochiensis* by describing the chromosome behavior in meiotic and

mitotic dividing cells (**chapter 1**). Subsequent marker analysis of progenies obtained by controlled crosses appeared to be less straightforward than usually applied for most plant and animal species. The biology of potato cyst nematodes involves many characteristics which obstructed linkage mapping in this organism. In **chapter 2**, an alternative mapping procedure is worked out for the construction of a genetic map. The linkage map of the potato cyst nematode included nine linkage groups and the molecular markers were shown to be evenly spread over the genome. The availability of a genomic map with a low kilobase/centimorgan ratio makes this species now amenable for map based cloning.

In contrast to the potato cyst nematode, a wealth of information is available on the genetics of potato and its relative tomato. In **chapter 3**, the inheritance of AFLP markers in potato was studied relative to already known RFLP, isozyme and morphological trait loci. Since AFLP markers appeared to be mapped as alleles rather than loci, this may have negative consequences for genetic mapping. As compared to more conventional RFLP markers, the identity of an AFLP (allele) is for the better part based on the mobility of an AFLP fragment. This potential disadvantage was largely compensated by the efficiency and sensitivity of the AFLP method. The allele specificity of AFLP markers was investigated in **chapter 4**. Different genetic maps of potato could be aligned on the basis of common AFLP markers and by linkage of common AFLP markers to chromosome specific RFLP markers, chromosome numbers can be identified for anonymous linkage groups. This feature provided an additional value to already mapped AFLP markers. Cataloging these AFLP markers potato by defining their mobility on autoradiogram images, provided a simple measure for alignment of genetic maps. Instead of the application of RFLP probes or designing specific PCR primers, map information can now be exchanged solely on the basis of the comparison of autoradiogram images. A description of the catalogue and its use for map alignment are outlined in **chapter 5**.

Applications of the AFLP catalogue are described in chapters 6 and 7. In **chapter 6**, monogenic resistance loci to *G. rostochiensis* (gene *H1*) and to *G. pallida* (gene *Gpa2*) are located on the potato map. The specificity of the *Gpa2* gene to a distinct set of *G. pallida* populations suggests a gene-for-gene relationship, similar to the interaction between *G. rostochiensis* and the *H1* gene. Chapter 7 describes a QTL approach towards mapping of a PCN resistance locus in potato. In contrast to the prevailing view that broad spectrum resistance to potato cyst nematodes should be regarded as a polygenically inherited trait, QTL analysis showed that the resistance to both species is for the better part ascribed for the action of a single locus *Grp1* (**chapter 7**). *Grp1* maps to a region on the potato map where other resistance genes to plant pathogens have been mapped. This finding indicates that major resistance genes are operating in complex inheriting resistance.

To clone *Gpa2*, a high resolution map for the region around this gene has been obtained (**chapter 8**). Interestingly, *Gpa2* is tightly linked to a major gene for resistance against potato virus X. Although both pathogen species are totally unrelated, the corresponding map region suggests that both genes are evolutionary related and may therefore be structurally homologous.

The implications of the results described in this thesis on the model of potato cyst nematode - potato interactions are discussed in **chapter 9**.

Chapter 1

Chromosome number of the potato cyst nematode *Globodera rostochiensis*

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ABSTRACT

The chromosome number of *Globodera rostochiensis* was determined by investigating: i) bivalent configurations at metaphase I, ii) meiotically reduced cells during oogenesis and iii) somatic mitosis at early embryonic cleavage divisions. A diploid chromosome number of 2n = 18 was found. Chromosome numbers were readily determined in polar bodies using the fluorochrome Hoechst 33258. An improved squashing method enabled observations at early cleavage divisions.

INTRODUCTION

The potato cyst nematode species *Globodera rostochiensis* (Woll.) Skarbilovich and *G. pallida* Stone are obligate plant parasites which are serious pests in potato crops. Populations of both species are classified into pathotypes as defined by their (in)ability to reproduce on a standard set of potato clones (Kort *et al.*, 1977). One of these clones, *Solanum tuberosum* spp. *andigena* CPC 1673, harboring the H_1-resistance gene, is used to define the *G. rostochiensis* pathotypes Ro_1 and Ro_4. A gene-for-gene relationship was demonstrated for the interaction between *G. rostochiensis* and the H_1-resistance gene. Virulence is inherited at a single locus and is recessive to avirulence (Janssen *et al.*, 1991). This gene-for-gene interaction is used as a model to unravel the molecular basis of virulence in the potato cyst nematode *G. rostochiensis*. Currently we are tracing molecular markers linked to the avirulence gene in backcross populations (Rouppe van der Voort *et al.*, 1994). Progress is restrained by serious gaps in the knowledge on the genetic constitution of this bisexual-reproducing cyst nematode species.

Data on the chromosome number of potato cyst nematodes are scarce and conflicting. Riley and Chapman (1957) observed nine pairs of chromosomes at first metaphase of meiosis (2n = 18) in *Heterodera rostochiensis*. It is noted that until 1970 (Jones *et al.*, 1970; Stone, 1973) both potato cyst nematode species were considered pathotypes of a single species, *H. rostochiensis*. Variations in the basic chromosome number of *H. rostochiensis* were reported by Cotten (1959, 1960). He observed diploid chromosome numbers of 20 and 22. Occasionally, 19, 23 and 24 chromosomes were found.

Whereas morphologically nearly indistinguishable, the two potato cyst nematode species are discriminated by 70% of their polypeptides as resolved by two-dimensional gel electrophoresis (Bakker & Bouwman-Smits, 1988). These data indicate that *G. rostochiensis* and *G. pallida* diverged millions of years ago. Hence, the discrepancy in chromosome numbers as reported by the afore-mentioned papers can be explained by the possibility that (a mix of) different species were analysed. Recently, the chromosome complement of one of the two species, *G. pallida*, was determined to be 2n = 18 although considerable variation was observed (Grisi *et al.*, 1995). The authors stated that part of this variation could be due to observational errors.

In this report, the chromosome number of *G. rostochiensis* was investigated. Using the fluorochrome Hoechst 33258 and an improved squashing method, the chromosome number was established by: i) observation of bivalent configurations at metaphase I, ii) the reduction process of the diploid complement during oogenesis and iii) determination of the re-established somatic number at early embryonic cleavage divisions.

MATERIALS AND METHODS

Populations and lines: The *G. rostochiensis* population "Mierenbos", classified as Ro_1 was obtained by the Plant Protection Service, Wageningen and multiplied in our laboratory. A virulent (Ro_5-22) and an avirulent (Ro_1-19) inbred line of *G. rostochiensis* were derived from controlled single matings and selected as described previously (Janssen

et al., 1990).

The *G. rostochiensis* lines were reared on the susceptible clone *S. tuberosum* spp. *tuberosum* L. cultivar "Eigenheimer". Potato plants were inoculated with approximately 200 cysts and placed in a growth chamber at 18°C and 16 h daylength. To harvest adult females, root balls were daily inspected. Young females outside the roots were collected with a small brush from the potato roots.

Fixation: Collected females were immediately fixed in cold 3:1 (v/v) ethanol-acetic acid and stored at -20°C for at least five days. This treatment caused increased permeability of the egg wall for cytological stains and facilitated squash preparations of eggs. Fixation was followed by either Hoechst 33258 or Feulgen staining.

Squash preparations and Hoechst staining: Microscope slides were pre-treated in a 1 % gelatin solution and, subsequently, air-dried. White females were placed on the slides, the adhering fixative was removed with tissue paper and a drop of 45 % (v/v) acetic acid was added. Females were opened with fine needles to release the body content. Gonads were squashed under a cover-slip (24 x 50mm) and the egg contents were spread. Slides were dipped in liquid nitrogen and, subsequently, the cover-slip was flipped off using a razor blade. The slides were air-dried, rinsed in 95 % (v/v) ethanol and fixed overnight in 3:1 (v/v) ethanol-acetic acid. Before staining, the slides were air-dried again and placed for 5 min in PBS (Sulston & Hodgkin, 1988). Preparations were stained for 5 min in 1 μg/ml Hoechst 33258 (bisbenzimide, Sigma) dissolved in PBS. Finally, the slides were rinsed in bidest.

Feulgen staining: After removing the adhering fixative, young females were incubated in 5N HCl for 25 min at room temperature. This results in a hydrolysis of the purine-deoxyribose bonds. Reactive aldehydes were demonstrated by Schiff's reagents (Feulgen & Rossenbeck, 1924). The incubation time was 3 h at 4°C. Thereafter, females were rinsed in tap water until the water remained clear.

Up to three stained females were placed on a slide, water was removed and a drop of 45 % (v/v) acetic acid was added. With fine needles the females were opened and the body contents was smeared on the slide. A cover slip was placed over it and pushed gently.

Microscopy: A Leitz Wetzlar Orthoplan microscope with a 100 x oil immersion objective was used to examine the Hoechst 33258 stained slides. The filter combination BP 355-425 / LP 460 was used to visualize the nuclei. Objects were photographed with a Kodak Ektachrome P800/1600 film adjusted at 400 ISO. The Feulgen stained nuclei and eggs were visualized with a 100 x oil immersion objective and photographed with Agfa Ortho film (25 ISO). Chromosome lengths were determined in Feulgen stained nuclei using a stage micrometer (Leitz Wetzlar).

RESULTS

The potato cyst nematode, *G. rostochiensis*, did not show intraspecific variation in chromosome behaviour during maturation divisions and embryogenesis among the populations tested. The data presented here are representative for the Ro_1-Mierenbos population and the lines Ro_1-19 and Ro_5-22.

Chromosome pairing at metaphase I: Chromosome pairing was clearly observed at diakinesis/metaphase I. At this stage the nuclear membrane disappeared. Nine bivalents of different sizes became discernable (Figure 1A). The chromosomes were rodshaped and lack visible constrictions. Each chromosome consisted of two separated chromatids which were arranged side by side, apparently perpendicular to the equatorial plane.

Reduction of the diploid complement during oogenesis: The first maturation division resulted in two haploid nuclei with a chromosome number of n = 9; the egg nucleus and the first polar nucleus, both with condensed chromosomes. The spindle was situated perpendicular to the cell membrane and the first polar nucleus ended near the periphery of the oocyte. The egg nucleus proceeded to anaphase II (Figs. 1B, 2A) whereas the first polar nucleus maintained its telophase I configuration (Figure 2B). The chromatids separated by parallel disjunction during anaphase II and no centromere was visible (Figure 1B). The second maturation division resulted in the formation of a second polar body and the egg pronucleus.

The amount of fluorescence reflected also the reduction of the diploid complement into the haploid condition (n = 9), which was accompanied with a change from 4C to 2C in the first meiotic division and to 1C in the second division (Figs. 1A, B, C). The sperm pronucleus which was localized at one pole of the oocyte (Figure 2C) moved towards the egg pronucleus and they fused to form the zygote nucleus. Polyspermy within one egg was never observed.

Somatic chromosome number at early embryonic cleavage divisions: Early cleavage divisions showed the restored somatic number of eighteen chromosomes (Figure 3). These mitotic chromosomes were much more elongated than the meiotic chromosomes and showed no primary constrictions. Both polar bodies remained visible during early embryonic development on top of the yolk, close to the egg wall (Figure 1C). While the first polar body, still with recognizable chromosomes persisted almost until completion of embryogenesis, the second polar body disintegrated soon after the first divisions. Different mitotic phases within one embryo indicated that daughter cells did not divide simultaneously from the first cleavage division onwards (Figure 1D).

Determination of chromosome number: The diploid chromosome number of *G. rostochiensis* was determined to be 2n = 18. Most observations of chromosomes were made on haploid nuclei of the first polar body (Figure 1E). The chromosomes of these nuclei were free from cytoplasm and were most definite as compared to other nuclear stages. Seven chromosomes varying in length from 0.8 to 1.2 μm could usually be

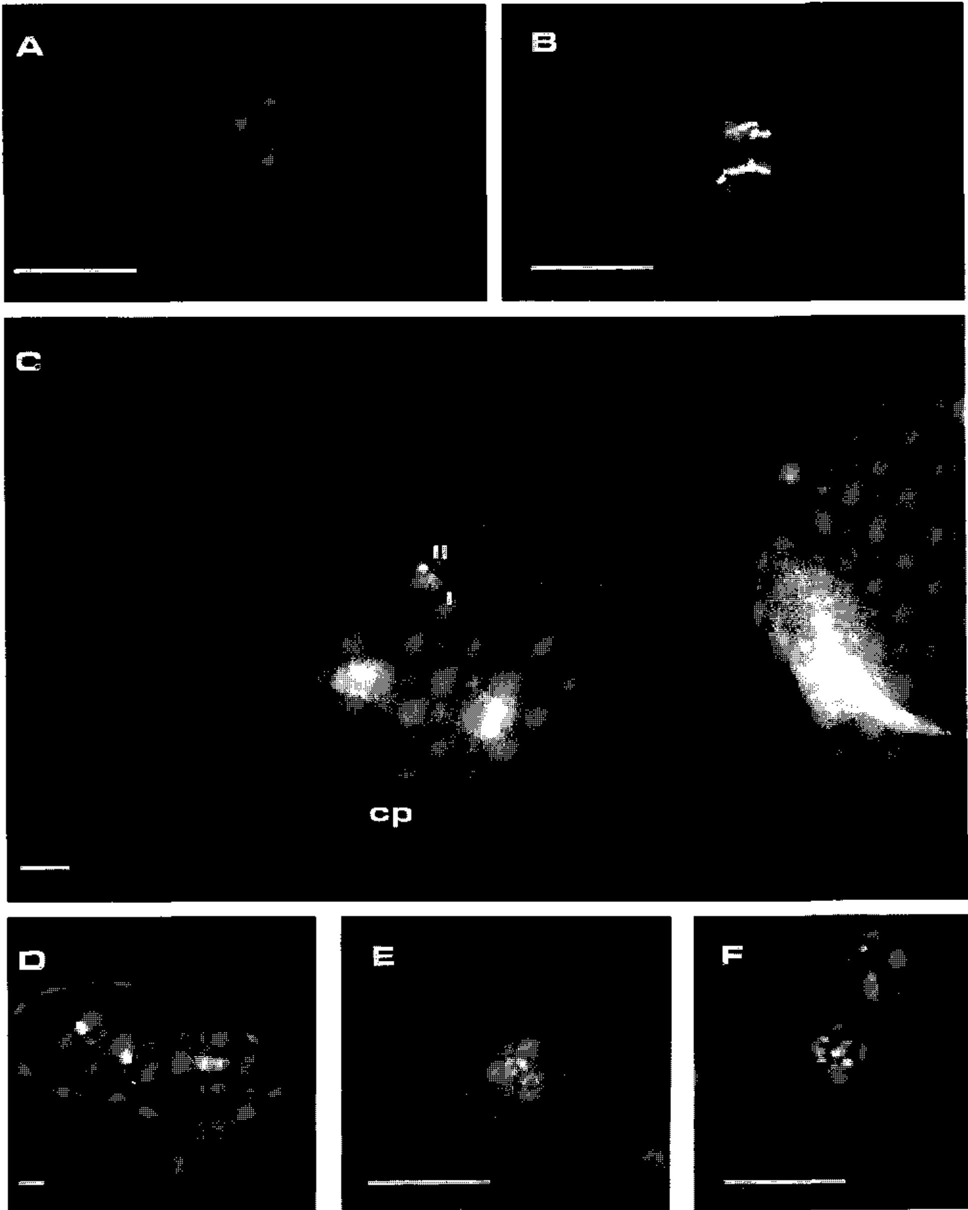

Figure 1: *Chromosomes and nuclei of Globodera rostochiensis stained with the fluorochrome Hoechst 33258. A: Metaphase I with nine bivalents; B: Anaphase II; C: First cleavage division (At both sides of the cleavage plate (cp) two somatic nuclei are discernible; the first (I) and the second polar body (II) are indicated); D: Second cleavage division; E: First polar nucleus, n = 9; F: First polar nucleus, n = 10. (Scale bar = 10 µm).*

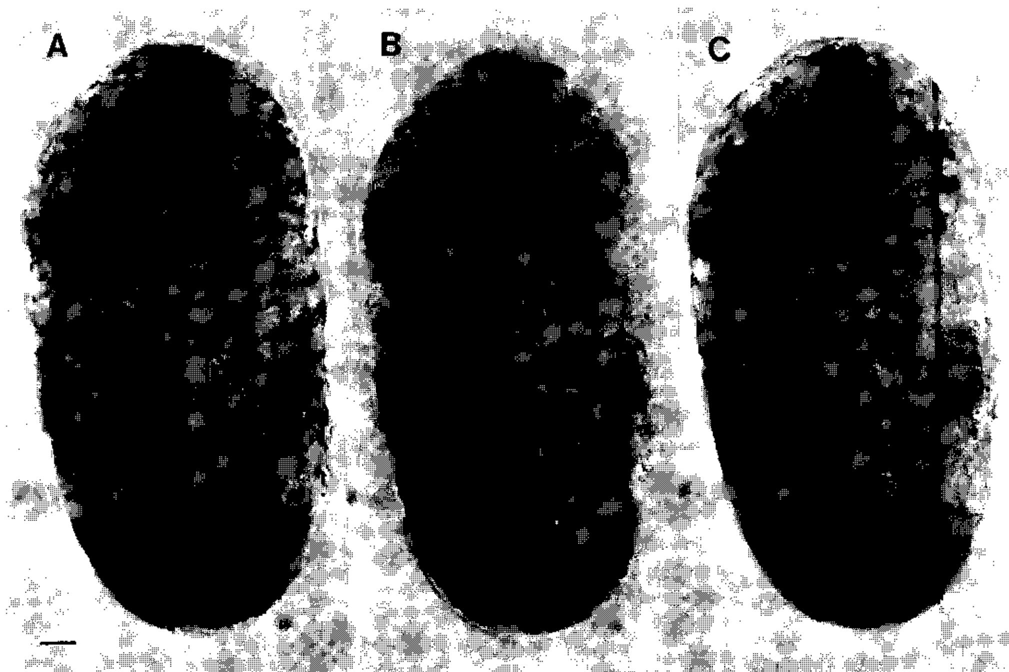

Figure 2: *A single oocyte at three focal planes with A: Anaphase II; B: First polar nucleus; C: Sperm pronucleus (Feulgen staining; only the objects marked with an arrow are Feulgen stained; Scale bar = 10 µm).*

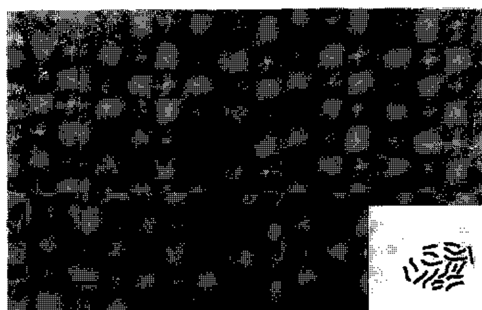

Figure 3: *Mitotic chromosomes at metaphase in a young embryo stained with Feulgen. The inset is a diagrammic representation; Scale bar = 10 µm.*

distinguished from a small chromosome (0.6 µm in length) and a large chromosome (1.5 µm in length). In only two out of the 200 polar nuclei examined, one extra chromosome was observed (Figure 1F).

DISCUSSION

Despite the extensive divergence at the molecular level between *G. rostochiensis* and *G. pallida* (Bakker & Bouwman-Smits, 1988; De Jong *et al.*, 1989; Folkertsma *et al.*, 1994) both species share a similar chromosome complement of 2n = 18. This chromosome number is analogous to karyotypes found in other cyst nematodes (genera *Globodera, Heterodera*), with the exception of *H. betulae* (Triantaphyllou, 1975). The karyotypic uniformity between *Globodera* species helps to explain the capacity of these extensively diverged species to produce interspecific hybrids. Matings between *G. rostochiensis* and *G. pallida* resulted in viable, although not fertile, second stage juveniles (Mugniéry, 1979; Mugniéry *et al.*, 1992).

Variation in the chromosome number of *G. rostochiensis* was much lower than the variation reported by Cotten (1959, 1960) for *H. rostochiensis* and by Grisi *et al.* (1995) for *G. pallida*. Only one percent of the polar nuclei showed a deviant chromosome number. Triantaphyllou (1975) observed in a small number of females of two *H. schachtii* Schmidt populations an extra chromosome which was transmitted to 50% of the progeny of these females. The extra chromosome divided normally during maturation divisions and was considered to be a supernumerary chromosome. However, the low frequency of an extra chromosome and because this extra chromosome can be the result of non-disjunction during anaphase II, it seems unlikely that supernumerary chromosomes are common in the *G. rostochiensis* populations studied.

The use of the fluorochrome Hoechst 33258 facilitated the counting of potato cyst nematode chromosomes. Hoechst 33258 interacts specifically with DNA. Its fluorescence increases the microscopic resolution and the chromosomes were mostly observed within one plane. Especially the first polar nuclei are suitable for karyotype determination. The absence of cytoplasmic counterstaining and the persistence of polar nuclei during embryogenesis enable unequivocal determinations of the haploid chromosome numbers. This is of potential use for karyotype analyses in other nematode species.

For a number of nematode species including *Caenorhabditis elegans, Meloidogyne hapla, Parascaris equorum* and *P. univalens*, chromosomes lacking a localized centromere are found (White, 1973; Goldstein & Triantaphyllou, 1980; Albertson & Thomson, 1982; Pimpinelli & Goday, 1989). Although by no means proven, the side by side arrangement of the chromatids at metaphase I, the parallel segregation at anaphase II and the absence of distinct centromeric constrictions suggest a holocentric nature of *Globodera* chromosomes.

It has been argued that the holocentric nature of nematode chromosomes may have been evolved to prevent cell death due to chromosome breakage (Pimpinelli & Goday, 1989). Characteristic for nematode genomes is their mosaic embryonic development in which each cell passes through a determined number of mitotic divisions and cannot be replaced by another cell. Breakages in monocentric chromosomes would be lethal to the cell and would thus affect the whole embryo. Holocentric chromosomes are characterized by a diffuse centromere which extends along the length of the chromosome. Fragmented holocentric chromosomes may still contain a spindle attachment site and remain through cell division. Holocentric chromosomes may provide nematodes a strategy to circumvent

lethal effects of chromosome fragmentation.

Finally, it is concluded that the chromosome number of the *G. rostochiensis* populations studied is 2n = 18 and constant. From strict bivalent pairing, observed in metaphase I, Mendelian segregation of maternal and paternal alleles is expected. This is of importance for a genetic analysis of the potato cyst nematode with molecular markers.

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Chapter 2

Linkage analysis by genotyping sibling populations: a genetic map of the potato cyst nematode using a "pseudo-F2" mapping strategy

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ABSTRACT

A mapping strategy is described for the construction of a linkage map of a non-inbred species for which individual offspring genotypes are not amenable to marker analysis. After one extra generation of random mating, the segregating progeny was propagated and bulked offspring populations were analysed. Although the resulting population structure is different from the one commonly used in mapping populations, we show that the maximum likelihood formula for a normal F2 is applicable for the estimation of recombination. This "pseudo-F2" mapping strategy in combination with the development of an AFLP assay on single cysts facilitated the construction of a linkage map of the potato cyst nematode *Globodera rostochiensis*. Using 12 pre-selected AFLP primer combinations, a total of 66 segregating markers were identified of which 62 mapped to nine linkage groups. These 62 AFLP markers are randomly distributed and cover about 65% of the genome. An estimate of the physical size of the Globodera genome was obtained by a comparative analysis with the number of AFLP fragments obtained from *Caenorhabditis elegans*. The methodology presented here resulted in the first genomic map of a cyst nematode. The low kilobase/centimorgan (kb/cM) ratio of the Globodera genome facilitates map based cloning of genes which mediate the plant-nematode interaction.

INTRODUCTION

Linkage maps are important tools in genetic research. High density maps including numerous molecular markers facilitate the genomic localization of monogenic and polygenic traits and have supported the cloning of genes for which no knowledge on the corresponding gene products was available. Both in population biology and in evolutionary studies, linkage maps have been used to identify genomic regions involved in selection processes. Moreover, linkage maps are of practical relevance for diagnostics, breeding and germplasm conservation.

In the last decade, detailed linkage maps based on restriction fragment length polymorphism (RFLP) markers have been developed for a number of species (reviewed in Tanksley *et al.* 1989; O'Brien, 1993). However, RFLP analysis involves labour intensive procedures and requires large quantities of DNA. With the advent of polymerase chain reaction-based molecular marker techniques such as randomly amplified polymorphic DNA (RAPD; Williams *et al.* 1990), sequence tagged micro satellites (STMS; Beckmann and Soller, 1990) and AFLP (Vos *et al.* 1995) more sensitive and time efficient methods became available for the generation of large numbers genetic markers. High density maps (> 500 markers) have been published for *e.g. Arabidopsis thaliana* (Reiter *et al.* 1992; Hauge *et al.* 1993), rice (Harushima *et al.* 1998), tomato and potato (Tanksley *et al.* 1992), barley (Qi *et al.* 1998), mice (Dietrich *et al.* 1996) and humans (Murray *et al.* 1994).

Linkage maps are preferably constructed by analysis of inbred or immortalized populations derived from the F1 of a cross between two homozygous inbred parents, *i.e.* RILs (recombinant inbred lines) or IF2 (immortalized F2; Gardiner *et al.* 1993). RILs and IF2s offer the advantage that the population can be propagated indefinitely thereby allowing for an unlimited number of markers and traits to be analyzed in replications in years and environments. Where litter sizes are small, data can be combined from several families and maps can be refined by collecting information from common databases. In plants, F2 and backcross (BC) populations are useful because many crops can be propagated clonally and backcrosses can be carried out without severe inbreeding depression. The benefit of molecular markers is that individual members of a population can be genotyped using minute amounts of tissue. Among the DNA marker systems, the AFLP technique is superior with respect to the outer detection limit. As little as 25 pg of genomic DNA is sufficient for the generation of a virtually unlimited number of markers (Vos *et al.* 1995). Still the isolation of such quantities can be an insurmountable problem for microscopic organisms that reproduce in a strictly outbreeding manner.

We set out to generate a genetic linkage map of the genome of the potato cyst nematode, a tiny roundworm which has so far only been analyzed at the population level. The potato cyst nematodes (PCN) *Globodera rostochiensis* and *G. pallida* are serious pests of potato accounting for estimated yield losses of 10% annually (Oerke *et al.* 1994). For growth and development PCN depend on a feeding site; a conglomerate of metabolically highly active plant cells. In resistant host plants, feeding site induction is hampered, resulting in a substantial reduction of the nematode population. As for many other plant parasites, a gene-for-gene relationship is postulated as a model for the interaction between cyst nematode genotypes and potato clones (Thompson, 1994; Bakker

et al. 1993). Several potato resistance genes have been identified and will probably be cloned in the near future (Leister *et al.* 1997; Van der Vossen *et al.* 1997). For the virulence of cyst nematodes, scarce genetic data are available. The ability to construct a genetic map is a prerequisite for the analysis of virulence traits of potato cyst nematodes.

By far the best characterized nematode genome is *Caenorhabditis elegans*, which map contains more than 1000 mutationally defined genes (Brenner 1974; Waterston *et al.* 1997). *C. elegans* has many attractive features for genetic research; it has a 3-day generation time, it reproduces either by self- or by cross-fertilization and strains can be grown on agar plates containing *Escherichia coli* as sole food source. These features are not applicable to the potato cyst nematode. Under natural conditions *G. rostochiensis* has one life cycle per year. It is an obligate biotrophic and amphimictic plant parasite. Inbred lines cannot be raised and a diapause seriously hinders mass rearings under laboratory conditions and the development of homozygous populations.

To circumvent our inability to analyze individual nematodes, a mapping procedure was developed based on the segregation analyses of a series of sibling populations. These sibling populations consisted of the encysted offsprings formed as a result of random mating. The strict physical separation of the offsprings in the cysts and the ability to analyze single cysts allowed us to follow the segregation patterns of molecular marker alleles.

As a consequence of the non-inbred nature of the offspring, the family of sibling populations does not compare to commonly used F2, BC1 and RIL families. Nevertheless, the maximum likelihood estimator of the recombination frequency was shown to be identical to the estimator normally used to analyze F2 populations. This novel strategy enabled the generation of linkage maps of non-inbred species in case individual genotypes are not amenable to marker analysis. Estimates of the physical and genetic size of the Globodera genome revealed a low kilobase/centimorgan (kb/cM) ratio. The described attributes are both necessary and sufficient for the isolation of genes involved in the intimate cyst nematode-plant interaction.

MATERIAL AND METHODS

Nematode crosses: The parental populations Ro1-19 and Ro5-22 were selected from controlled matings using individuals of the natural populations Ro1-Mierenbos and Ro5-Harmerz, respectively (Janssen *et al.* 1990). Females were obtained by inoculating pre-parasitic juveniles separately on the roots of *Solanum tubersosum* cv. Eigenheimer grown in Petri dishes on water agar. For PCN sex is determined epigenetically, and females will develop if the inoculum density is low, *viz.* one second stage juvenile per root tip. Males were reared in pots and extracted from the soil with an Oostenbrink elutriator (Oostenbrink, 1960). Controlled matings were performed in Petri dishes as described by Mugniery (1982). The plates were kept at 21°C. Four to six weeks after inoculation bright, white-colored females were crossed by placing one male on the gelatinous matrix of the female.

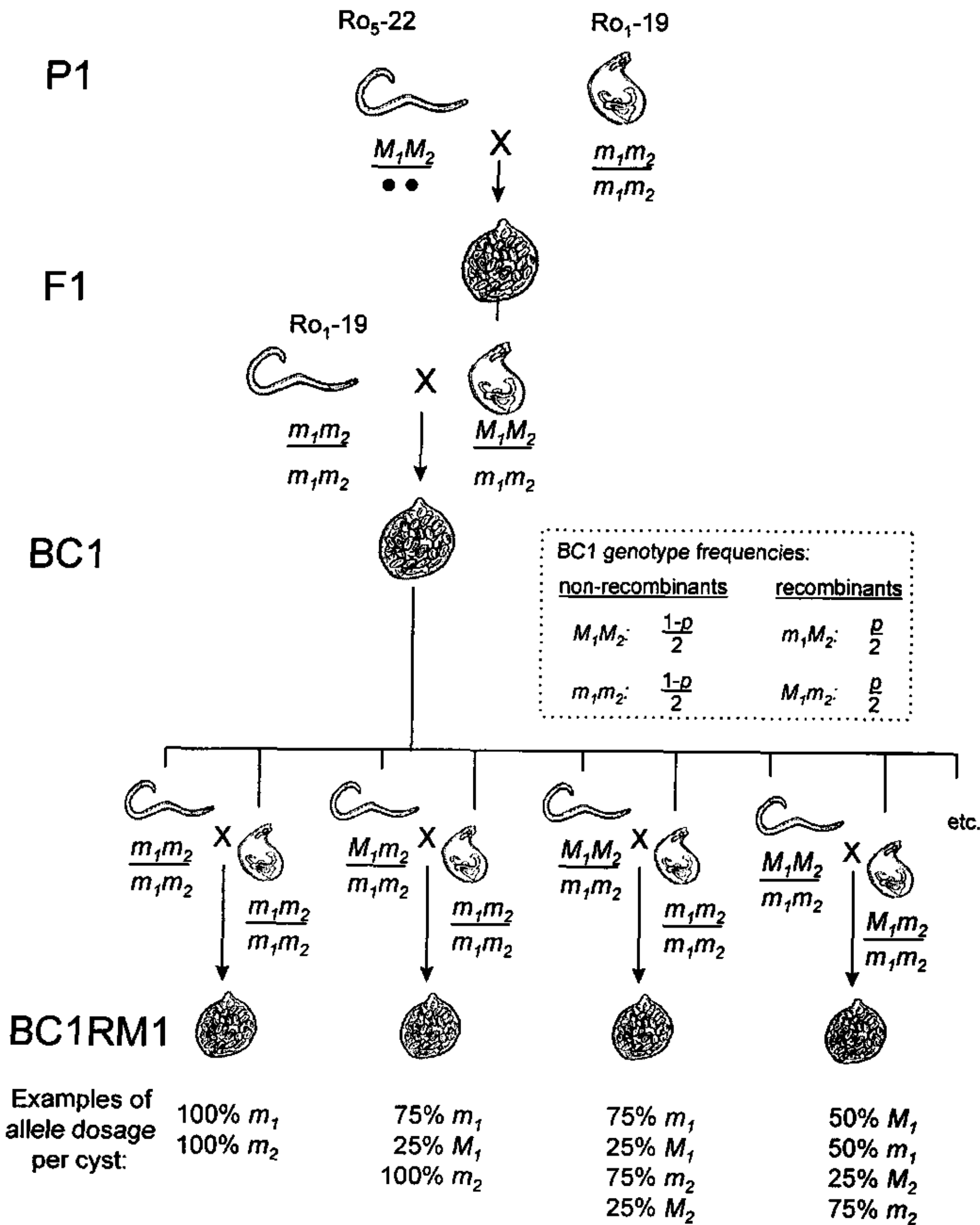

Figure 1: *Crossing strategy to produce a BC1RM1 progeny. The pear-shaped objects represent female nematodes, the males are vermiform and cysts containing numerous eggs are indicated by globular objects. The genotypes for the hypothetical loci M1 and M2 are indicated. A controlled mating between a Ro1-19 female and a Ro5-22 male (generation P1) resulted in an F1 cyst for which the F1 females were backcrossed with a Ro1-19 male. A random mating between the BC1 nematodes generated the BC1RM1 family of sibling populations. Recombination between markers M_1 and M_2 is estimated on the basis of the marker phenotypes observed in the BC1RM1 cysts, where M_1 and M_2 are parent Ro5-22 specific markers, linked in coupling phase.*

Two months later dark-brown cysts were collected and stored at 4°C for at least 9 months. Although the diapause can be circumvented by avoiding desiccation of the cysts (Janssen *et al.* 1987), we encountered a reduction in the reproduction rate if we immediately used Ro_1-19/Ro_5-22 hybrids for production of F1 females.

The mating scheme (Figure 1) involved two generations of controlled crosses and one generation of random mating. The first generation consisted of a controlled mating performed between a male of population Ro_5-22 and a female of population Ro_1-19 (generation P1). For the second generation, an F1 hybrid female was backcrossed with a Ro_1-19 male. As individual nematodes cannot be analyzed separately, the BC1 population hatched from the cyst was propagated in a pot containing the susceptible potato cultivar Eigenheimer. A random mating was assumed between the individuals of the BC1 population during this multiplication step. The result of each mating was a cyst. Thus, the family of sibling cyst populations consists of BC1RM1 cysts.

The BC1RM1 family used in this study consisted of 107 cysts; the highest number among a set of 36 multiplied BC1 cysts. Before production of the BC1RM1 progeny, the numbers of eggs within a BC1 cysts were counted to determine the multiplication rate for each BC1 cyst. Out of a BC1RM1 progeny of 107 cysts, 56 individual cysts allowed successful preparation of AFLP template. Hence, the mapping population is now represented by 56 BC1RM1 cysts.

Estimates of recombination frequencies: To estimate the frequency of recombination between the hypothetical markers M_1 and M_2 in the meiosis of the F1 hybrid female, a mating table was constructed (Table 1). As a consequence of the use of a dominant marker system in a non-inbred species, the linkage phase of the markers is variable. Knowledge of the linkage phase is essential for proper estimation of the recombination frequency. To assure that all AFLP alleles segregating in the BC1RM1 family are in coupling phase a backcross was involved. As a result of the backcross, only the Ro_5-22 specific markers account for the mating table.

After the backcross, the maternal alleles were transmitted to the BC1 genotypes. These BC1 genotypes mated randomly and, depending on the BC1 genotype, up to four different gametes were produced. In contrast to the analysis of single genotypes, the frequencies of the separate gamete types were irrelevant. A BC1RM1 cyst phenotype represents the phenotype of the bulked offspring of a single mating, and is determined by the combination of the paternal and maternal gamete pools. As a consequence, the frequency of the bulked gamete phenotypes are the same as the genotype frequencies of these (BC1) parents. By multiplying the BC1 genotype frequencies, the formulas for expected frequencies of the BC1RM1 phenotypes were obtained (Table 2). These formulas are similar to the formulas which belong to a cross of the type $M_1M_2/m_1m_2 \times M_1M_2/m_1m_2$ with marker M_1 and M_2 in coupling phase (Allard, 1956). Solving the maximum likelihood equation (Appendix I) shows that the estimate of the recombination frequency and the corresponding information function (Appendix II) for this mating scheme are similar to the formulas which belong to a cross of the type $M_1M_2/m_1m_2 \times M_1M_2/m_1m_2$

with marker M_1 and M_2 in coupling phase (Allard, 1956). It is concluded that despite of the aberrant design of the crosses, recombination frequencies can be estimated as described for an F2 population.

Table 1: Mating table for the BC1RM1 mapping population to derive the estimator of the recombination frequency p for a family of BC1RM1 cysts. M_1 and M_2 are visible alleles of loci *M1* and *M2* for which m_1 and m_2 are null alleles. Because the BC1RM1 phenotypes represent the bulked offspring of a single mating, the BC1 genotype frequency is similar to the (pooled) gamete frequency. By multiplying the BC1 genotype frequencies for each parental genotype combination, the probabilities for the BC1RM1 cyst phenotypes are obtained (see Table 2).

BC1 genotypes				M_1M_2/m_1m_2	m_1m_2/m_1m_2	M_1m_2/m_1m_2	m_1M_2/m_1m_2
	BC1 genotype frequency			$\frac{1-p}{2}$	$\frac{1-p}{2}$	$\frac{p}{2}$	$\frac{p}{2}$
		BC1 phenotypes		M_1M_2	m_1m_2	M_1m_2	m_1M_2
			Gametes	M_1M_2, m_1m_2, M_1m_2, m_1M_2	m_1m_2	M_1m_2, m_1m_2	m_1M_2, m_1m_2
				BC1RM1 cyst phenotypes			
M_1M_2/m_1m_2	$\frac{1-p}{2}$	M_1M_2	M_1M_2, m_1m_2, M_1m_2, m_1M_2	M_1M_2	M_1M_2	M_1M_2	M_1M_2
m_1m_2/m_1m_2	$\frac{1-p}{2}$	m_1m_2	m_1m_2	M_1M_2	m_1m_2	M_1m_2	m_1M_2
M_1m_2/m_1m_2	$\frac{p}{2}$	M_1m_2	M_1m_2, m_1m_2	M_1M_2	M_1m_2	M_1m_2	M_1M_2
m_1M_2/m_1m_2	$\frac{p}{2}$	m_1M_2	m_1M_2, m_1m_2	M_1M_2	m_1M_2	M_1M_2	m_1M_2

Table 2: Expected and observed frequencies for the occurrence of BC1RM1 cyst phenotypes; p_j is the probability for the occurrence of the marker phenotype j where p_j is a function of the recombination frequency p. The formulas for p_j can be obtained by multiplying the corresponding BC1 genotype frequencies depicted in Table 1. These formulas are similar as obtained for the expected frequencies of marker phenotypes in an F2 population with alleles M_1 and M_2 in coupling phase (Allard, 1956).

Rec. frequencies	*BC1RM1 cyst phenotypes*			
	M_1M_2	m_1m_2	M_1m_2	m_1M_2
p_j	$\frac{1}{4}(3-2p+p^2)$	$\frac{1}{4}(1-p)^2$	$\frac{1}{4}p(2-p)$	$\frac{1}{4}p(2-p)$
complete linkage *(p = 0)*	3	1	–	–
absence of linkage *(p=0.5)*	9	1	3	3

DNA manipulations: DNA was extracted from single cysts as follows. A clean, acetonated cyst was transferred to a fixation dish (Seinhorst 1962) placed in a humid chamber and soaked in 75 µl milliQ water overnight. The cyst was crushed to release the eggs/J2 juveniles and the cyst wall was discarded. The mixture was spun down for 90 sec. and the pellet was washed twice with 100 µl milliQ water. The pellet was frozen for 30 min at -80°C and thawn at room temperature. This freeze-thawn procedure was repeated twice. Subsequently, 200 µl proteinase K buffer (0.1M Tris/HCl pH 8.5, 0.1M EDTA, 0.5% SDS) and five µl Proteinase K (20 ng/µl) were added and this mixture was incubated at 50°C for 4 h or overnight. After one phenol and one chloroform/isoamylalcohol extraction, the DNA was precipitated by adding 480 µl 96% ethanol, 40 µl 0.1 M sodium acetate and incubated at -20° for 1 h. The mixture was centrifuged for 10 min at 13,000 rpm. The pellet was washed in 70% ethanol, dried and dissolved in 20 µl TE (Sambrook *et al.* 1989). For AFLP template preparation, 10 µl of DNA was used, and 3 µl fresh 5 × restriction-ligation buffer (Vos *et al.* 1995), 0.1 µl *Eco*RI (2.5 U), 0.12 µl *Mse*I (2.5 U) and 1.78 µl milliQ water was added. After incubation at 37° C for 1 h, an adapter-ligation mix was added containing 0.5 µl of the *Eco*RI and the *Mse*I adapter and the AFLP procedure was continued as described (Vos *et al.* 1995).

For the selective amplification of restriction fragments, "*Eco*RI-primers" (E-primers) and "*Mse*I-primers" (M-primers) were used (Vos *et al.* 1995). A total of four or five selective nucleotides for each primer pair was used indicated by the primer codes "E+2/M+2" and "E+2/M+3" respectively.

Genomic DNA from the parental populations was isolated from approximately 6000 young females, harvested 5-6 weeks after inoculation (Roosien *et al.* 1993). AFLP template preparation was performed using 50 ng of this DNA (Vos *et al.* 1995).

Construction and analysis of the genetic map: Linkage analyses of pairwise recombination frequencies between the markers were calculated by use of the computer program JoinMap version 1.4 (Stam, 1993). The data were analyzed according to an F2 segregation type and marker genotypes were encoded either "C" (presence of a marker) or "A" (absence of a marker). Markers which were linked to other markers at LOD values between 1.0 and 3.0 were subjected to a chi-square test of independence by means of 2×2 contingency tables. These tests were applied by use of the program Linkage-1 (Suiter *et al.* 1983). Markers were considered to be linked if the chi square test criterion exceeded 8.0 (Ellis *et al.* 1992). To correct for spurious linkage due to skewness of segregation, this test was also applied for linked markers which deviated from the expected segregation ratio of 3:1. A chi-square test is more conservative as compared to the log-likelihood method since the production of 2×2 contingency tables result in two additional degrees of freedom to test for significant linkage. Recombination frequencies were converted into map units (cM) by use of the Kosambi function. Graphic representations of the maps were made by the computer program Drawmap (Van Ooijen, 1994)

Clustering of markers was tested by a chi-square test for goodness of fit at $P \geq 0.05$ (Rouppe van der Voort *et al.* 1997).

Estimation of genome coverage: An estimate of the genetic length of *G. rostochiensis* was obtained by use of the method of Hulbert *et al.* (1988). This method is based on calculation of the probability P that a pair of linked markers randomly chosen from a genome of size G, has a LOD score $\geq$ a threshold value T. An estimate of P is given by the obtained number of marker pairs at LOD = T and the theoretical number of marker pairs for a given number of marker loci. The genome size G follows from the expression $2x/P$ where x is the genetic distance obtained from the expected LOD score. An MS Excel spreadsheet for obtaining genome size estimates by use of this method is available upon request.

Estimate of physical properties of the *Globodera* genome: Estimates of the physical characteristics of the *G. rostochiensis* genome were obtained by analysis of the number of DNA fragments amplified with a given primer combination in comparison with the number of DNA fragments obtained from the *C. elegans* genome. For *C. elegans*, AFLP fragments were generated from the strain N2 Bristol (kindly provided from H. van Luenen, The Netherlands Cancer Institute, Amsterdam, The Netherlands) by using 64 primer combinations. Thirty-two primer combinations were obtained with AFLP primers E+GA or E+AA and the *Mse*I primers having two selective nucleotides at their 3' end. Another 32 combinations were made between primer E+GA and all possible M+3 primers. The effects of the species, the number of selective nucleotides and G+C content of the primer extension on the total number of AFLP fragments amplified were analyzed by multiple linear regression (Genstat 5 release 3.2, Payne *et al.* 1987).

RESULTS

Selection of AFLP markers: AFLP markers specific for parental population Ro5-22 were selected by testing 70 E+2/M+2 and E+2/M+3 primer combinations on template DNA of populations Ro5-22 and Ro1-19. Because the AFLP template is based on a population of nematodes where markers can have an allele frequency as low as $p = 0.25$ (Figure 1), a third template, *viz.* a 9:1 mixture of Ro1-19/Ro5-22 template DNA was included to discard Ro5-22 specific AFLP markers which may not give a clear amplification product in a genetic background of Ro1-19 alleles. An example of a primer combination pre-screen and AFLP genotyping of the progeny is shown in Figure 2.

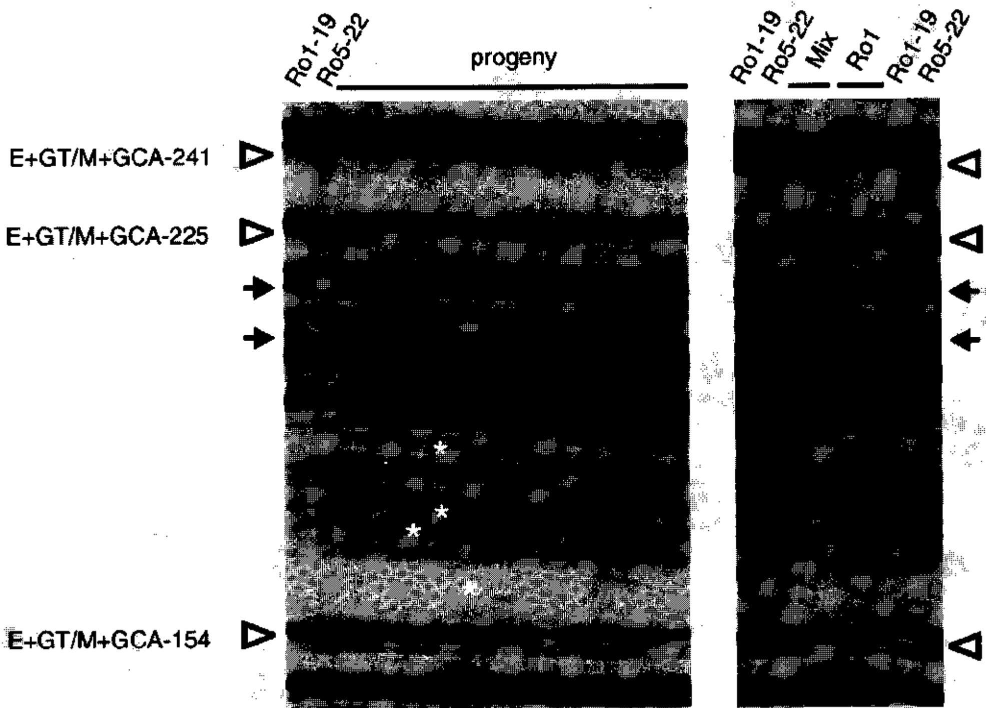

***Figure** 2: Part of an autoradiogram showing AFLP fingerprints generated using primer combination E+GT/M+GCA. On the left, AFLP markers amplified from the parental lines Ro1-19 and Ro5-22 and a subset of the BC1RM1 progeny. Ro5-22 specific markers E+GT/M+GCA-241, E+GT/M+GCA-225 and E+GT/M+GCA-154 are indicated. Ro1-19 specific markers, present in all BC1RM1 progeny cysts are indicated by small arrows. Template independent fragments are marked by an asterisk. On the right, AFLP profiles generated from the parental lines, two 9:1 Ro1-19/Ro5-22 template mixtures and two templates of field population Ro1-Mierenbos to screen for reproducible amplification at low allele frequencies of Ro5-22 specific markers.*

Out of a total of 7104 DNA fragments amplified from population Ro5-22, about 7% of these fragments was absent in Ro1-19; which is on average 7.6 Ro5-22 markers per primer combination (SD=3.6). About 60% of these Ro5-22-specific markers could either not readily be amplified from the 9:1 template mixture or not reliably be scored due to co-migrating fragments. Taking this into account, twelve AFLP primer combinations were selected which amplified at least three Ro5-22 markers per primer combination.

Using these primer combinations, 66 segregating AFLP markers were reproducibly amplified from the BC1RM1 progeny. Fifteen AFLP markers did not segregate because of their presence in all BC1RM1 cysts. This may be the result of the coincident presence of a Ro5-22 marker allele in the Ro1-19 F1 male or a strongly skewed segregation ratio towards Ro5-22 alleles. Eighteen AFLP markers could not be recovered from the progeny cysts, despite their presence in the 9:1 mixtures. This could be explained if it is assumed that these markers (1) were not transmitted by the P1 Ro5-22 male (Figure 1), (2) were subject to a strong negative selection or (3) could not reliably amplified from the BC1RM1 cysts. The latter case is not inconceivable because below an absolute threshold of approximately 25 pg DNA as starting material, amplification products may remain under the detection level (Vos *et al.* 1995). An indication that the protocol for single cyst AFLP involves a template preparation which is on the verge of the sensitivity of the AFLP technique derives from the observation of template independent amplification products in some of the AFLP profiles of single cysts (Figure 2). AFLP fingerprints which involve templates prepared from minute amounts of genomic DNA, may contain template independent amplification products (Vos *et al.* 1995).

Construction and analysis of the genetic map: At a first glance the BC1RM1 mapping population would require different estimates of recombination frequencies as those commonly used for analyzing F2 or BC1 mapping populations derived from inbred parents (Figure 1). However, evaluation of the frequencies at which the different marker phenotypes can occur (Table 1) revealed that recombination frequencies can be estimated using the Maximum likelihood estimator for two markers in coupling phase segregating in an F2 population. Therefore, the dataset could be analyzed by JoinMap as an F2 population type.

Out of the 66 segregating markers, 62 (94%) were incorporated into nine linkage groups. Four markers could not be assigned to any linkage group. This number of linkage groups corresponded to the haploid number of chromosomes of *G. rostochiensis* (Riley and Chapman, 1957; Rouppe van der Voort *et al.* 1996). The linkage groups were established by stepwise lowering the LOD score from 8.0 to 2.0. Seven linkage groups could be discriminated at LOD = 6.0. Lowering the linkage threshold to 3.0 resulted in the addition of two linkage groups (Figure 3). Expansion of the map was observed without agglomeration of linkage groups.

The nine linkage groups of the *G. rostochiensis* map range in size from 20 cM to 85 cM with a total map distance of 431 cM. The average distance between the markers is 8 cM as determined by the dividing the total map distance by the number of markers minus the number of linkage groups. Clustering of markers as tested by a chi-square test for goodness of fit was not observed.

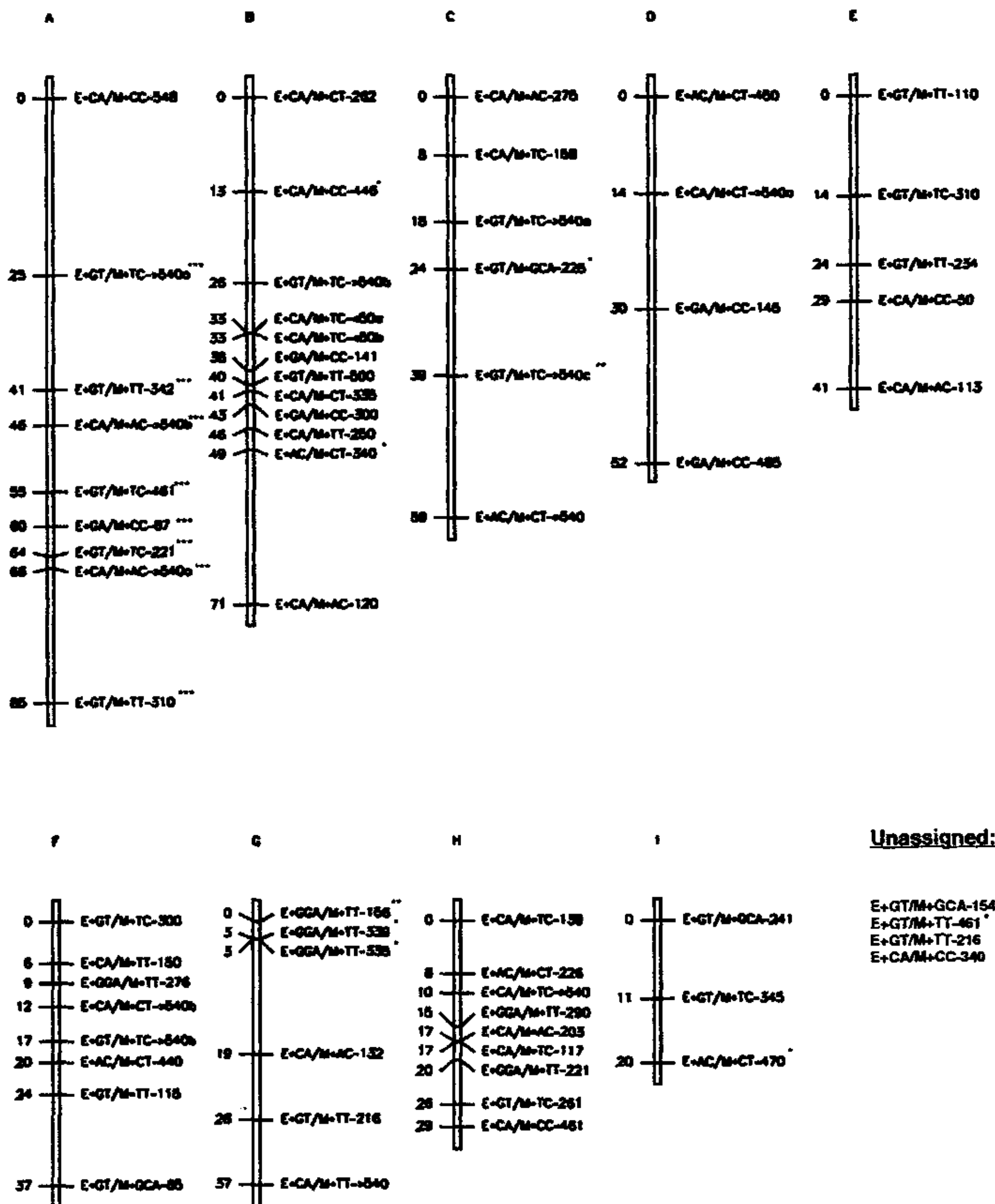

Figure 3: Genetic linkage map of G. rostochiensis. Markers are designated by the enzyme combination (E and M), followed by the selective nucleotides (+NNN) and a relative measure for the molecular weight of the AFLP fragment. All of the markers are mapped with a LOD score > 3.0, except for five markers (underlined) which were mapped with LOD scores between 1.0 and 3.0. These markers were assigned to linkage groups by means of a chi-square test of independence with a chi-square value above 8 (Ellis et al. 1992). Markers that deviated from the expected 3:1 segregation are marked with asterisks (: deviation at 0.05 > P > 0.01, **: deviation at 0.01 > P > 0.001, ***: deviation at P << 0.001). Four markers remained unassigned.*

The assumption of random mating in the BC1 generation was based on the supposition of monogamy. This supposition was tested by investigating the level of segregation distortion of the markers. Since 75% of the progeny was expected to contain a Ro5-22 marker (Figure 1), insemination of one female by multiple males would tend to increase the fraction of the progeny bearing such a Ro5-22 marker. Deviations from the expected Mendelian ratio were observed for 19 (29%) of the segregating markers (tested by a X^2 test at $P < 0.05$). However only 8/19 of the skewed segregating marker loci showed a bias towards the presence Ro5-22 alleles. These markers mapped primarily on two regions of the genome. On linkage group *A*, Ro5-22 alleles were under-represented, possibly due to selection. On this linkage group, segregation ratios varied between 1:1 and 8:1 where a 1:3 ratio for absence vs. presence of an AFLP marker was expected. For three markers on linkage group *G*, Ro5-22 markers were over-represented; with segregation ratios varying between 1:6 and 1:8. Therefore no genetical evidence was obtained that for the fertilization of single PCN females by multiple males.

Genome coverage: The finding that virtually all markers mapped to one of the linkage groups indicated that this set of markers cover a significant proportion of the potato cyst nematode genome. An estimate of the maximum genetic length of the genome was obtained using the method of Hulbert *et al.* (1987). Since no clustering was observed on the map all 66 markers were taken into consideration. With a mapping population of 56 genotypes, 253 marker pairs were found within 34 cM distance at LOD = 1.0 whereas 170 marker pairs within a distance of 26 cM were linked with LOD = 3.0. This corresponds to estimates of G = 629 cM and G = 676 cM respectively. Taking these data together, the genome size of *G. rostochiensis* is likely to approximate 650 cM. This implies that the markers presented here cover about 65% of the potato cyst nematode genome.

Estimates of genome size and G+C base composition: Estimates of the physical properties of the *G. rostochiensis* genome were obtained by a comparative analysis with the *C. elegans* genome. Theoretically, the total number of AFLP fragments obtained per primer combination depends on the number and the G+C content of the selective nucleotides. Since the physical size and the G+C base composition of the *C. elegans* genome are known (Sulston and Brenner, 1974), a comparative AFLP analysis may provide a relative measure of the *G. rostochiensis* genome.

In Figure 4, numbers of amplification products obtained with varying G+C contents in the selective nucleotides are presented for both nematode species. For *C. elegans* strain N2 Bristol, a total of 6773 AFLP fragments were amplified using a similar set of primers as has been used to analyze the *G. rostochiensis* genome. AFLP fingerprints were not taken into account, when fragment numbers differed more than 3 × SD from the mean number of fragments for a given G+C content of the primer extension. Such extreme differences were infrequently observed and are presumably caused by the depletion or

abundance of particular sequence motifs. A multiple linear regression analysis showed that both the number of selective nucleotides and the G+C content of the extension had a significant effect on the numbers of AFLP fragments amplified (at $P < 0.001$, determined by a *t*-test). Similar intercepts (genome size) and slopes (G+C content) of the regression lines of both species were obtained for both primer extension lengths (Figure 4). This indicates that the estimated size and the average G+C composition of the *G. rostochiensis* genome are similar to the estimates obtained for the *C. elegans* genome, *viz.* 8×10^7 bp for haploid size genome and a G+C content of 36% (Sulston and Brenner, 1974).

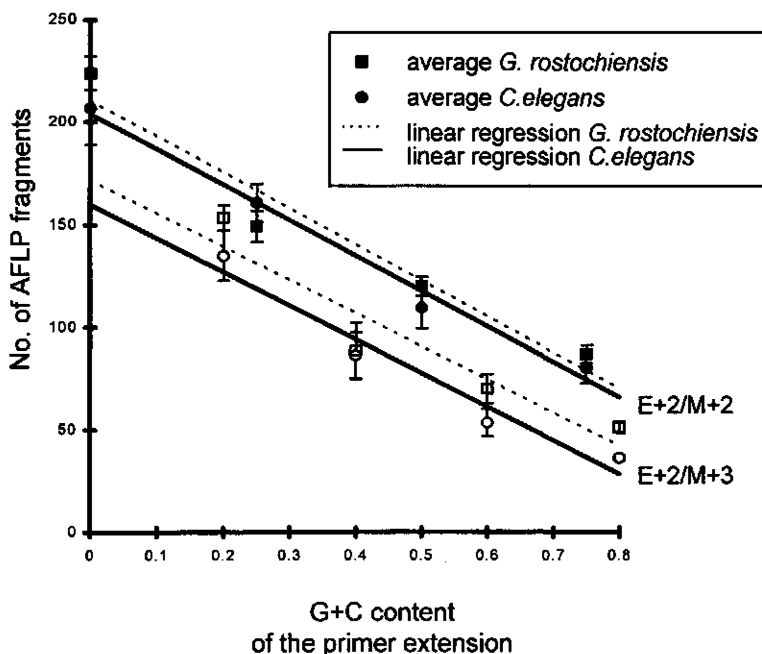

Figure 4: *Multiple linear regression of the species effect, G+C content and number of selective nucleotides in the AFLP primer on the number of AFLP fragments obtained. The similar intercepts and slopes of the regression lines for both types of primer combinations indicate a similar genome size and G+C content of G. rostochiensis relative to that of C. elegans.*

DISCUSSION

A linkage map of the potato cyst nematode was constructed by means of a "pseudo F2" mapping strategy. This novel approach enabled us to generate a linkage map of a non-inbred species for which individual genotypes are not amenable to extensive marker analysis. We have chosen the name "pseudo F2" because the experimental design for

analysis of a family of sibling populations allowed for the use of same estimator of recombination frequencies as applied in a normal F2. The mapping procedure differed from an F2 in at least three aspects. Firstly, the mapping was not based on recombination in both male and female meiosis, but only in the meiosis of the female F1 hybrid. Secondly, a backcross was involved to assure that all AFLP alleles segregating in the BC1RM1 were in the same linkage phase. Thirdly, data on marker segregation were derived from the analysis of sibling populations instead of individual genotypes.

Segregation analysis of markers in families of sibling populations can be performed by use of F2 estimators if: (1) random mating is assumed, (2) parental specific markers are identified and (3) the linkage phase of these markers is known. Linkage analysis between dominant markers in coupling phase in an F2 is straightforward. A simulation study on the informativity of an F2 population has shown that for two dominantly segregating markers in coupling phase, linkage can reliably be detected for recombination frequencies up to 0.2, even within a progeny of about 50 in size (Maliepaard *et al.* 1998). Moreover, the benefit of an F2 estimator of recombination frequencies is the use of the algorithms in existing mapping software.

The level of polymorphism between the parental populations appeared to be sufficient for construction of a genetic map. Per AFLP primer combination, an average of seven Ro5-22 specific amplification products were observed. However, since only one linkage phase could be analyzed for segregation, a substantial part of the Ro5-22 specific AFLP markers could not be used in the described experimental design. In addition, the amplification of Ro5-22 AFLP markers from the offspring bulks may be hampered by the occurrence of competing priming sites derived from the Ro1-19 genome and/or differences in allele frequencies of the marker in the bulks. At segregating marker loci, allele frequencies of 0.25 and 0.5 (Figure 1) should be clearly detectable. To ensure a reliable discrimination between the presence or absence of a Ro5-22 marker allele in a bulked BC1RM1 progeny, a control lane was included comparable with an allele frequency of 0.1. This pre-screen revealed 12 selected AFLP primer combinations by which 66 segregating markers were identified in the BC1RM1 family. In retrospect, for reasons of stronger selectivity in the amplification reaction, a higher efficiency in obtaining informative primer combinations would be achieved when more E+2/M+3 primer combinations were included. Hence, the generation of a saturated map of *G. rostochiensis* will primarily be based on the use of E+2/M+3 primer combinations.

The presupposition of monogamy was justified since no overall bias was detected for the occurrence of Ro5-22 alleles in the population. A nematode feature which may prevent insemination of one female by multiple males is the formation of a so-called copulatory plug which prevents multiple inseminations (Barker, 1994). "Plugging" and "non-plugging" races have been found for *C. elegans* in which males of a plugging race cause the formation of gelatinous material over the vulva of the recipient female after mating (Hodgkin and Doniach, 1997). For other genera of nematodes, copulatory plugs have been described as well (Baird *et al.* 1994). However, the presence of a gelatinous

matrix on the vulva of virgin PCN females makes it difficult to establish whether PCN females produce a copulatory plug after mating.

The segregation distortion encountered may be explained by the differential viability of the segregating genotypes. Before production of the BC1RM1 progeny, the numbers of eggs for 36 BC1 cysts were counted (average 420 eggs/cyst (SD = 171)) and compared with the numbers of new BC1RM1 cysts. The BC1RM1 progeny sizes ranged between 0 and 114 with an average of 35 (SD = 35). Also within a BC1RM1 population, differences in cyst contents were observed which largely determined the success rate of the AFLP template preparation. The largest, most healthy BC1RM1 family was selected for further analysis. Out of a total of 107 cysts, only 56 cysts allowed for the preparation of AFLP template. Cysts with the lowest egg contents invariably failed for the production of AFLP template. Differences in viability between BC1RM1 cysts may be the result of inbreeding depression; an inbreeding coefficient of 5/16 applies for the BC1RM1 family. Janssen *et al.* (1992) observed a similar reduction in the vitality of the selected populations Ro_1-19 and Ro_5-22 as compared to F1 juveniles derived from controlled matings between these populations. Inbreeding depression may be caused by the combination of lethal (recessive) alleles. Likely genomic positions for such loci are located on linkage group *A* which harbors the most skewed segregating marker loci. Selection processes may also occur during the life cycle of the nematode to favor certain genotypes to grow under the artificial laboratory conditions.

The linkage map of the *G. rostochiensis* genome includes 62 markers which are randomly distributed over the genome. Four markers could not be assigned to any genomic region. The 62 markers are separated into nine linkage groups, a number similar to the basic chromosome number of the potato cyst nematode (Riley and Chapman, 1957; Grisi *et al.* 1995; Rouppe van der Voort *et al.* 1996). The random distribution of markers on the map indicates a homogenous distribution of recombination events along the nine chromosomes. This random distribution of markers on the map is in contrast to severe clustering of markers observed on the maps of many plant genomes (*e.g.* Tanksley *et al.* 1992; Kesseli *et al.* 1994; Rouppe van der Voort *et al.* 1997). Clustering of markers is often ascribed to recombination suppression in centromeric regions (Sherman and Stack 1995). The behavior of chromatid separation during meiosis suggests that the *G. rostochiensis* chromosomes lack a localized centromere (Rouppe van der Voort *et al.* 1996) which may explain the random distribution of the markers along the map.

Based on the marker segregation data, a rough estimate of 650 cM was obtained for the total genetic length of the *G. rostochiensis* genome. The physical size of the *G. rostochiensis* genome was estimated by a comparative analysis of the number of AFLP fragments produced using the *C. elegans* genome as a reference. The size and G+C content of the *C. elegans* genome has been determined by DNA renaturation kinetics (Sulston and Brenner, 1974) and will be exactly known when the genome sequencing project is finished. Similar numbers of bands per fingerprint were obtained irrespective of the experimental conditions applied. This suggests that the haploid genome size and G+C content for both nematodes are highly comparable. These estimates on the physical

properties of the *G. rostochiensis* genome are not much different to estimates obtained for the genome of the related plant parasitic nematode species *Meloidogyne* (Pableo *et al.* 1988; Pableo and Triantaphyllou, 1989). The estimates on the physical and genetic size of the Globodera genome reveal an overall ratio of 120 kb of DNA per 1 cM distance.

The ability to construct a genetic map for the potato cyst nematode opens the way for map-based cloning in this organism. The genome size is small, and sufficient recombination occurs to allows for fine mapping any gene of interest. A proportion of 120 kb/cM implies that the equivalent of one unit of recombination corresponds to a DNA fragment length which can now readily be cloned into bacterial artificial chromosomes (BACs; Shizuya *et al.* 1992; Zhang *et al.* 1996). A translation of units of recombination into distances in base pairs can be in conflict with regional differences in recombination frequencies. However, the random distribution of markers on the *G. rostochiensis* map observed indicates that the major part of the genome is accessible for isolating genomic clones to construct an overlapping contig.

The coding capacity of nematode DNA and the fact that the majority of nematode genomes analyzed so far comprise a low proportion of repetitive DNA (Hammond and Bianco, 1992), should facilitate the construction of BAC contigs. Potato cyst nematodes are an important pest of potato to which a number of resistance genes are now in the process of being cloned (Ganal *et al.* 1995; Leister *et al.* 1997; Van der Vossen *et al.* 1997). The approach described in this paper opens to way to identify the nematode counterparts with which the resistance gene products interact. For example, a similar set-up would in principle allow for the identification of *e.g.* *AvrHs-1*$^{pro-1}$ in the beet cyst nematode *Heterodera schachtii* which counterpart, the sugar beet resistance gene *Hs1*$^{pro-1}$ was recently cloned (Cai *et al.* 1997).

ACKNOWLEDGEMENTS

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Appendix I

An estimate of the recombination frequency p between markers M_1 and M_2 could be obtained by solving the maximum likelihood equation (*MLE*) for p (Fischer, 1921):

$$MLE(p) = \sum_j Z_j \frac{1}{p_j} \frac{\delta p_j}{\delta p} = 0$$

where p_j are the probabilities for the occurrence of the marker phenotypes j (Table 1b) and Z_j are the observed frequencies for these marker phenotypes. The corresponding standard error can be derived from the information function I_p (Allard, 1956). Since full estimates (P) of p have been derived for all possible allelic configurations in parents of diploid crosses (Allard 1956; Ritter et al. 1990; Ritter and Salamini, 1996; Maliepaard et al. 1998) our aim was to obtain the appropriate estimate for p and corresponding standard error. According to the gamete types which occur in the F1 females a mating table was constructed (Table 1). From this mating table the formulas for p_j were derived from for the gamete combinations which can occur in the BC1 cyst (Table 2). In table 3, a calculation table is presented according to Ritter et al. (1990) to derive the maximum likelihood equation and the corresponding information function.

Table 3: Calculation of the maximum likelihood equations (MLE) and corresponding information function (I_p) from the marker class distributions obtained in Table 1.

Phenotypes	p_j	$\frac{\delta p_j}{\delta p}$	$\frac{1}{p_j}\frac{\delta p_j}{\delta p}$	$\frac{1}{p_j}\left(\frac{\delta p_j}{\delta p}\right)^2$	Z_j
M_1M_2	$\frac{1}{4}(3-2p+p^2)$	$-\frac{1}{2}+\frac{1}{2}p$	$\frac{2(p-1)}{3-2p+p^2}$	$\frac{(1-p)^2}{3-2p+p^2}$	a
M_1m_2	$\frac{1}{4}p(2-p)$	$\frac{1}{2}-\frac{1}{2}p$	$\frac{2(p-1)}{p(2-p)}$	$\frac{(1-p)^2}{p(2-p)}$	b
m_1M_2	$\frac{1}{4}p(2-p)$	$\frac{1}{2}-\frac{1}{2}p$	$\frac{2(p-1)}{p(2-p)}$	$\frac{(1-p)^2}{p(2-p)}$	c
m_1m_2	$\frac{1}{4}(1-p)^2$	$-\frac{1}{2}+\frac{1}{2}p$	$\frac{-2}{1-p}$	1	d
Sum	1	0	for MLE	for I_p	n

From Table 3 follows that the maximum likelihood equation is given by:

$$\frac{2a(p-1)}{3-2p+p^2}+\frac{2b(1-p)}{p(2-p)}+\frac{2c(1-p)}{p(2-p)}+\frac{2d}{1-p}=0$$

$$\Leftrightarrow (1-p)\left\{\frac{b+c}{p(2-p)}-\frac{a}{p^2-2p+3}\right\}-\frac{d}{1-p}=0$$

$$\Leftrightarrow (1-p)\left\{\frac{(b+c)(p^2-2p+3)-ap(2-p)}{p(2-p)(p^2-2p+3)}\right\}-\frac{d}{1-p}=0$$

$$\Leftrightarrow (1-p)^2\left\{(b+c)(p^2-2p+3)-ap(2-p)\right\}-dp(2-p)(p^2-2p+3)=0$$

Assume $Q = 1\text{-}p$:

$$Q^2\left\{(b+c)(Q^2+2)+a(Q+1)(Q-1)\right\}+d(Q^2+1)(Q-1)(Q^2+2)=0$$

$$\Leftrightarrow Q^2\left\{(b+c)(Q^2+2)+a(Q^2-1)\right\}+d(Q^2-1)(Q^2+2)=0$$

Assume $S = Q^2$ (thus $S = (1\text{-}p)^2$):

$$S\left\{(b+c)(S+2)+a(S-1)\right\}+d(S-1)(S+2)=0$$

$$\Leftrightarrow (a+b+c)S^2+(2b+2c-a)S+dS^2+dS-2d=0$$

$$\Leftrightarrow (a+b+c+d)S^2+(2b+2c-a+d)S-2d=0$$

$$\Leftrightarrow S=\frac{-(2b+2c+d-a)\pm\sqrt{(2b+2c+d-a)^2+4(a+b+c+d)2d}}{2(a+b+c+d)}$$

For $n=a+b+c+d$

and $X=\dfrac{a-2b-2c-d}{2n}$:

$$S=X\pm\sqrt{X^2+\frac{2d}{n}}\Rightarrow S_1=X+\sqrt{X^2+\frac{2d}{n}}\wedge S_2=X-\sqrt{X^2+\frac{2d}{n}}$$

Since $S = (1\text{-}p)^2$:

$p=1\pm\sqrt{S_1}\wedge p=1\pm\sqrt{S_2}$

Because $p \in <0,1>$ and $\dfrac{2d}{n}>0$ (which makes $S_2<0$): the estimate P from p is given by: $P=1-\sqrt{S_1}$

This equation is similar to the recombination frequency estimate obtained for a cross of the type: $M_1M_2/m_1m_2 \times M_1M_2/m_1m_2$ as calculated by Allard (1957) and Ritter et al. (1990)

and Ritter et al. (1996). It is noted that the formula presented in Ritter et al. (1996) contains a few typesetting errors.

Appendix II

The standard error of the estimate P can be derived from the information function I_P. From table 3 follows that $I_P = n\left(\frac{(1-p)^2}{3-2p+p^2} + \frac{(1-p)^2}{p(2-p)} + \frac{(1-p)^2}{p(2-p)} + 1\right)$

$$\Leftrightarrow I_p = n\left(\frac{2p-5p^2+4p^3-p^4}{p(3-2p+p^2)(2-p)} + \frac{6-16p+16p^2+8p^3+2p^4}{p(3-2p+p^2)(2-p)} + \frac{6p-7p^2+4p^3-p^4}{p(3-2p+p^2)(2-p)}\right)$$

$$\Leftrightarrow I_p = \frac{2n(3-4p+2p^2)}{p(3-2p+p^2)(2-p)}$$

which is the I_P function an F2 population as presented in Allard (1957), Ritter et al. (1990) and Ritter et al. (1996).

Chapter 3

The inheritance and chromosomal localisation of AFLP markers in a non-inbred potato offspring

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ABSTRACT

AFLP is a new technique to generate large numbers of molecular markers for genetic mapping. The method involves the selective amplification of a limited number of DNA restriction fragments out of complex plant genomic DNA digests using the PCR reaction. With six primer combinations 264 segregating AFLP amplification products were identified in a diploid backcross population from non-inbred potato parents. The identity of an AFLP marker was specified by the primer combination of the amplification product and its size estimated in bases. The segregating AFLP amplification products were mapped by using a mapping population with 217 already known RFLP, isozyme and morphological trait loci. In general the AFLP markers were randomly distributed over the genome, although a few clusters were observed. No indications were found that AFLP markers are present in other parts of the genome than those already covered by RFLP markers. Locus specificity of AFLP markers was demonstrated because equally sized amplification products segregating from both parental clones generally mapped to indistinguishable maternal and paternal map positions. Locus specificity of AFLP amplification products will allow to establish the chromosomal identity of linkage groups in future mapping studies. Since AFLP technology is a multi-locus detection system, it was not possible to identify the AFLP alleles which belong to a single AFLP locus. The consequences of a genetic analysis based on single alleles, rather than on loci with two or more alleles on mapping studies using progenies of non-inbred parents are discussed.

Introduction

Molecular markers are indispensable tools for generating genetic linkage maps and have provided a major contribution to the genetic knowledge of many cultivated plant species (reviewed in Tanksley et al. 1989). In addition to being of basic importance to genetic and evolutionary studies, molecular markers are useful to localise monogenic and polygenic traits which allows the efficient introgression and selection of individuals with specific characteristics.

Genetic maps of potato, *Solanum tuberosum*, have been constructed using restriction fragment length polymorphisms (RFLPs; Bonierbale et al. 1988; Gebhardt et al. 1989; Gebhardt et al. 1991; Jacobs et al. 1995; Kreike, 1995). Co-dominant and locus specific RFLP markers are unequalled for many applications. However, markers techniques which employ the polymerase chain reaction (PCR) (reviewed by Rafalski et al. 1993) are rapid, technically simple and require only small amounts of DNA. Unfortunately, the reproducibility of random amplified polymorphic DNA (RAPD) analysis is often poor due to low annealing temperatures and other reaction conditions. The dominant inheritance of RAPD markers is a disadvantage, particularly in mapping populations derived from non-inbred parents. Therefore, RAPD markers have not been used up till now for the construction of a genetic map in potato. Drawbacks related to RAPDs can be circumvented by, *e.g.*, the locus-specific sequence-tagged-site approach (STS; Olsen et al. 1989). Alternatively, sequence-tagged micro-satellite (STMS) primers can be used (Beckmann and Soller, 1990; Provan et al. 1995).

Recently, a reliable and efficient method has been developed to identify a large number of molecular markers (Vos et al. 1995). The method, called AFLP is a new PCR-based technique for DNA fingerprinting. Prior to PCR-amplification genomic DNA is digested by two restriction enzymes. Subsequently, oligonucleotide adapters are ligated to the resulting restriction fragments to generate template DNA for PCR. Template DNA is amplified using PCR primers complementary to the adapter sequences. Addition of extra nucleotides at the 3' end of the PCR primers allows the selective amplification of only those restriction fragments starting with nucleotides homologous to those of the primers. In this way AFLP fingerprints can be tailored to produce patterns of desired complexity. In principle, both the restriction enzymes and the nature and number of selective nucleotides can be varied. Consequently, a virtually unlimited number of markers can be generated. The reproducibility of AFLP profiles is assured by using primer sequences of at least 16 nucleotides at stringent amplification conditions. The reproducibility of the AFLP technique is further demonstrated by the consistently found relative band intensities. The quantitative differences in band intensities between the lanes, are easily used to recognize the homo- or heterozygous presence of an AFLP marker allele.

In this article the usefulness of the AFLP technique is investigated. A relatively well-characterized mapping population of potato (Jacobs et al. 1995) was used to study the localisation of AFLP markers relative to 197 RFLP, 9 isozyme and 11 morphological

markers. The various segregation patterns of AFLP amplification products observed in the offspring from non-inbred parents was used to evaluate the inheritance of putative AFLP loci. The findings on the inheritance of AFLP markers in potato are generalized for other mapping studies in progenies of non-inbred parents. The AFLP markers mapped in this study may enable the identification and mapping of co-migrating AFLPs from other genotypes and thereby facilitate future mapping studies.

Material and Methods

Plant material: A mapping population of 68 diploid potato genotypes was analyzed, descending from the cross USW5337.3 × 77.2102.37 (Jacobs et al. 1995). The male parent 77.2102.37 is a descendant of VH^34211 × USW5337.3. Consequently the mapping population is a backcross. As a result of the relatedness of the parental clones every locus of clone 77.2102.37 has one allele which is identical by descent to an allele of clone USW5337.3.

The AFLP protocol: DNA isolation was performed on frozen leaf tissue using the method as described by Van der Beek et al. (1992). The AFLP method was performed essentially as described by Vos *et al.* (1995), and is briefly outlined below. The method involves four steps: (1) template preparation, (2) selective pre-amplification of primary template with PCR primers having one 3' selective nucleotide, (3) selective amplification with ^{33}P-labelled PCR primers having three 3' selective nucleotides, and (4) separation of labelled fragments on polyacrylamide sequencing gels followed by autoradiography.

1. Template preparation

Primary template DNA was prepared in a one step restriction-ligation reaction. Approximately 0.5 mg genomic DNA was digested for four hours at 37°C using 10 U *Eco*RI, 5 U *Mse*I and 8 ml 5× restriction-ligation buffer (50 mM Tris.HAc pH7.5, 50 mM MgAc, 250 mM KAc, 25 mM DTT and 250 ng/ml BSA) in a final volume of 40 ml. After one hour 10 ml ligation mixture was added, containing 5 pMol biotin-labelled adapter fitting the *Eco*RI site, 50 pMol *Mse*I adapter, 0.1 ml 10 mM ATP, 1.0 U T4-DNA ligase and 2 ml 5× restriction-ligation buffer.

The sequences of the adapter fitting the *Eco*RI site was:

```
5'-biotin-CTCGTAGACTGCGTACC
                    CTGACGCATGGTTAA-5
```

The sequence of the *Mse*I adapter was:

```
5'-GACGATGAGTCCTGAG
      TACTCAGGACTCAT-5
```

Selection of biotinylated DNA restriction fragments having *Eco*RI/*Eco*RI or *Eco*RI/*Mse*I ends was performed by binding to streptavidin coated Dynabeads M-280 (Dynal, Oslo, Norway). Before usage, the beads were washed once in 10 volumes STEX (1 M NaCl, 10 mM Tris pH 8.0, 1 mM EDTA, 0.1% (v/v) Triton X-100) and resuspended in 5 volumes of 2×STEX. Per sample 50 ml resuspended beads were added to a final volume of 100 ml. The suspension was incubated for 30 min. at room temperature. Gentle agitation was applied to ensure proper binding of biotinylated DNA. The beads were collected with a magnet (Dynal MPC). The supernatant with discarded and the beads were washed three times in 100 ml STEX, and each time new reaction vials were used. After the final wash step, the beads were resuspended in 200 ml $T_{0.1}$E-buffer (10 mM Tris pH 8.0, 0.1 mM EDTA) and stored at 4°C.

2. Selective pre-amplification of primary template DNA

The first PCR amplification of adapter-ligated restriction fragments (primary template) has the purpose to generate large quantities of secondary template DNA suitable for AFLP reactions with radioactively labelled selective primers. The primer sequence appropriate to prime the *Eco*RI end, with one additional 3' nucleotide (E+1 primer), used in selective pre-amplification reaction was: 5'-GACTGCGTACCAATTC*A* (E+A). The sequences of the selective M+1 primers fitting the *Mse*I ends were: 5'-GATGAGTCCTGAGTAA*A* (M+A) and 5'-GATGAGTCCTGAGTAA*C* (M+C). Five ml of primary template (bead suspension) was mixed with 30 ng E+A and 30 ng M+A or M+C primer, 0.8 ml 5 mM dNTPs, 0.08 U *Taq* polymerase (Perkin Elmer, USA), 2 ml 10×PCR-buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$) in a final volume of 20 ml. PCR reactions were performed in a PE-9600 thermal cycler (Perkin Elmer, Norwalk, USA) using the following touch-down PCR profile: 14 cycles 30 s at 94°C, 30 s at 65°C and 60 s at 72°C. Per cycle, the annealing temperature was decreased by 0.7°C. The initial cycles were followed by 24 cycles of 30 s at 94°C, 30 s at 56°C and 60 s 72°C. To verify the successful production of secondary template, electrophoresis of 10 ml of the amplification reaction should give a smear on a ethidium bromide stained agarose gel. Secondary template was diluted 25-fold in $T_{0.1}$E and stored at 4°C.

3. Selective Restriction Fragment Amplification (SFRA)

For the reproducible and selective amplification of a limited number of DNA restriction

fragments primer combinations were used with three additional 3' nucleotides (E+3 and M+3 primers). Such primer pairs would in theory recognize only one out of 4096 of the initial number of restriction fragments. The following E+3 primers were used in combination with M+3 primers were used in the AFLP mapping experiment: E+AAA (5'-GACTGCGTACCAATTC*AAA*), E+AAC, E+ACA, E+AGA or E+ATG, in combination with M+ACG (5'-GATGAGTCCTGAGTAA*ACG*), M+CAC, M+CAG, M+CAT or M+CTC. Whereas all these E+3 primers can anneal to secondary template produced with E+A, the first M+3 primer will only anneal to secondary template produced with M+A. The other four M+3 primers require secondary template produced with M+C. The PCR reaction mixture was composed of 5 ml secondary template, primed with 5 ng ^{33}P end-labelled E+3 primer and 30 ng unlabelled M+3 primer, whereas the other components and the temperature profile was as described above. The following six primer combinations were used: E+AAA/M+ACG, E+AAC/M+CAC, E+AAC/M+CAG, E+ACA/M+CAC, E+AGA/M+CAT and E+ATG/M+CTC.

4. Separation of labelled fragments and autoradiography

Reaction products were loaded on a 5% polyacrylamide gel in 1×TBE electrophoresis buffer (Sambrook et al. 1989), using a Bio-Rad (Richmond, USA) sequence gel system. The gels were dried on Whatmann 3MM paper and X-ray films (Konica, Tokyo, Japan) were exposed for 1-4 days at room temperature. To store digitized images of the AFLP profiles an alternative method was performed by Keygene N.V., Wageningen. After fixing and drying of the gels on the glass plate, the radioactive signal was scanned using a Fuji BAS-2000 phosphor-image analysis system (Fuji Photo Film Company Ltd, Japan).

Nomenclature of AFLP markers: Designation of AFLP markers was based on the primer combination used and the size of the amplification products. The primer combinations are abbreviated by the letters of the three selective nucleotides at the 3' end of the E+3 primer before, and a M+3 primers behind the slash. The approximate sizes of the amplification products, expressed in nucleotides was estimated on the basis of the SequaMark 10 base ladder (Research Genetics, Huntsville, AL, USA). It is noted that the mobility of amplification products also depends on their nucleotide composition.

Data analysis: In Figure 1, the different types of Mendelian segregation patterns of AFLP amplification products, found in the offspring of non-inbred parents, are given. In case an amplification product was found only in one parental clone and segregated as a presence/absence polymorphism in the offspring, the underlying genetic model is assumed to be *Aa* × *aa* or *aa* × *Aa* (Figure 1, model 6 and 8, respectively). In case comigrating amplification products were found in both parental clones which segregated as a band intensity polymorphism in the offspring the underlying genetic model is assumed to be *Aa* × *AA* or *AA* × *Aa* (Figure 1, model 2 and 4, respectively). In case both types of

Figure 1: Segregation of AFLP amplification products in the progeny from non-inbred parental genotypes.

Genetic model	Mendelian Ratio	AFLP Phenotypes on autoradiogram		JoinMap Code	Remarks
		parents	offspring		
1. *AA* × *AA* → *AA*	-			-	not segregating
2. *AA* × *Aa* → *AA*, *Aa*	1:1 ♂			H × B	segregation data included in paternal dataset
3. *AA* × *aa* → *Aa*	-			-	not expected in backcross
4. *Aa* × *AA* → *AA*, *Aa*	1:1 ♀			A × H	segregation data included in maternal dataset
5. *Aa* × *Aa* → *AA*, *Aa*, *aa*	1:2:1			H × H	segregation data included in both datasets
6. *Aa* × *aa* → *Aa*, *aa*	1:1 ♀			A × H	segregation data included in maternal dataset
7. *aa* × *AA* → *Aa*	-			-	not expected in backcross
8. *aa* × *Aa* → *Aa*, *aa*	1:1 ♂			H × B	segregation data included in paternal dataset
9. *aa* × *aa* → *aa*	-			-	not visible

polymorphisms (presence/absence and band intensity) were observed among offspring phenotypes descending from parents with the weaker band intensity phenotype the underlying genetic model is assumed to be according to the model *Aa* × *Aa* (Figure 1, model 5). Mapping data were obtained by visual interpretation of autoradiograms, independently by two persons. In most cases the zygosity differences in band intensity polymorphisms could be scored unambiguously. Concurrent observation of presence/absence as well as the band intensity polymorphisms allowed to establish the codominant 1:2:1 segregation which is much more informative in linkage mapping than the dominant 3:1 segregation. In lanes where presence of a band was clear, but zygosity was ambiguous, the computer program JOINMAP, VERSION 1.4 (Stam, 1993) offered a solution. JOINMAP allows not only genotype codes for homozygous (A), heterozygous (H) or alternative homozygous observations (B), but also for non-A (C), i.e. H or B and non-B (D), i.e. either A or H. The rigid distinction between a 3:1 segregating presence/absence polymorphism or a 1:2:1 segregation is therefore futile in the situation of Figure 1, model 5.

Observed segregation ratios were analyzed for deviation from Mendelian ratios using the Chi-square test for goodness-of-fit. It should be noted that up till now only segregation patterns of amplification products were characterized. Putative allelic relations between amplification products were ignored.

Map construction: Map construction using JOINMAP 1.4 (Stam, 1993) was according to Van Eck (1995) and Jacobs et al. (1995). JOINMAP 1.4 is not designed to analyze linkage in a mapping population from non-inbred parents. Therefore, the data structure had to be modified. In brief, the method consists of the conversion of one dataset on offspring genotypes into two datasets on female and male gamete segregation. Separate datasets used for estimation of female and male recombination frequencies allows the construction of independent female and male linkage groups. The different segregation types, shown in Figure 1, should be assigned to the appropriate dataset and indicated by the correct JOINMAP segregation codes. Polymorphic amplification products segregating from the

female parent (Figure 1, model 4 and 6) are indicated by the JOINMAP code 'A × H'. The code 'H × B' is used to indicate the polymorphisms segregating from the male parent (Figure 1, model 2 and 8). Codominant AFLP markers segregating in a 1:2:1 ratio were indicated with the code 'H × H' (Figure 1, model 5). The A × H and H × H encoded AFLP markers were assigned to the maternal dataset and the H × H and H × B to the paternal dataset. Linkage groups were identified by stepwise lowering the LOD score from eight to three. A LOD score of at least three was used to identify the 24 maternal and paternal linkage groups. Identification of linkage groups could be troublesome in case erroneous linkage between markers from different chromosomes induced fusion of linkage groups. Erroneous linkage, caused by skewed segregation or type I errors (falsely accepted null-hypothesis), was identified and corrected when the marker concerned did not show proper linkage to flanking markers belonging to the other linkage group. The chromosome maps shown in this article are the result of merging those female and male linkage groups. Differences in marker distance and marker order along the male and female map were mainly caused by random sampling differences and could be corrected in the final map by feeding fixed sequence files to JOINMAP prior to merging the separate linkage groups. Alternative marker orderings along the chromosome map with comparable likelihoods could be reached by JOINMAP, and contradictions of the final marker order with those marker orders already established in the male and female map were precluded. Maps were drawn with the aid of the graphical package DRAWMAP (Van Ooijen, 1994) in orientations homologous to tomato and potato maps of Tanksley et al. (1992).

Digitized images of the AFLP profiles of the parental clones are available from the world wide web at URL: www.spg.wau.nl/pv/aflp/catalog.htm.

RESULTS

Selection of primer combinations: The diploid parents of a potato mapping population, USW5337.3 and 77.2101.37 were tested against a large number of E+3 / M+3 primer combinations (data not shown). The majority of these primer combinations generated AFLP profiles with on an average one hundred amplification products per lane. The better part of the primer combinations generated suitable and highly polymorphic AFLP profiles. However, in some instances profiles were rather complex, as they comprised up to 150 bands. Other primer combinations resulted in profiles disturbed by single excessively amplified, possibly repetitive sequences. Six primer combinations were chosen on the basis of the high number of polymorphisms found between the parental clones of this mapping population, as well as the number of polymorphic amplification products shared with another mapping population (Rouppe van der Voort et al.(1997a). Between the parental clones the number of polymorphic amplification products visible on the gel, ranged between 29 and 35 per primer combination. Figure 2 shows parental AFLP profiles

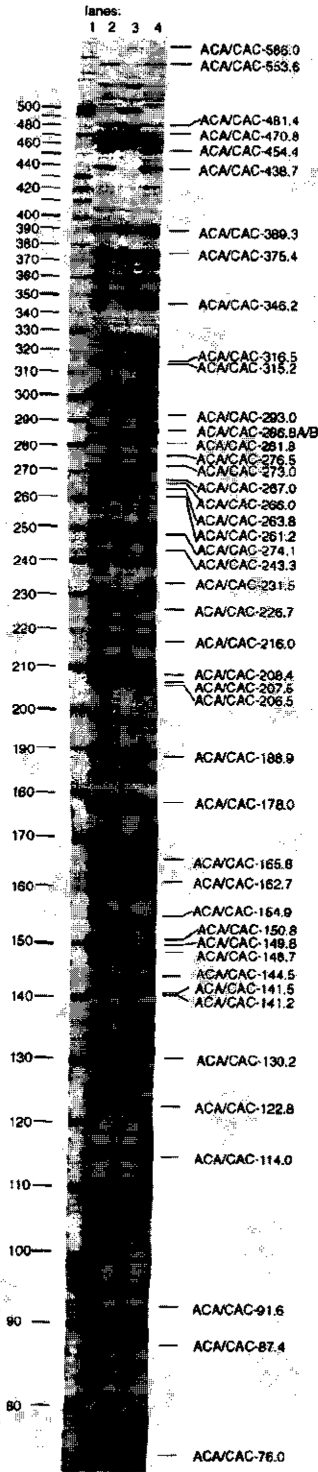

Figure 2: Image of the parental AFLP profiles generated with primer combination E+ACA/M+CAG. Segregating amplification products which were mapped on the potato genome (Figure 3) are indicated in this figure. The names are based on primer extensions and fragment lengths. Lane 1: 10 base ladder; lane 2: internal size marker (generated by using AFLP template of four potato genotypes); lane 3: female clone USW5337.3; lane 4: male clone 77.2102.37

generated with primer combination E+ACA/M+CAC. Polymorphic amplification products which were mapped on the potato genome (Figure 3) are indicated in this figure.

Identification of segregating amplification products: The numbers of segregating amplification products are presented in Table 1. These numbers, ranging between 38 and 49 polymorphisms per primer combination, now also include the 1:2:1 and the 1:1 intensity polymorphisms as they were initially not detectable on the basis of the parental phenotypes. Based on the number of polymorphisms relative to the total number of bands per individual, the average heterozygosity of the parents USW5337.3 and 77.2102.37 was 34% and 40% respectively. The actual heterozygosity will be somewhat higher, because closely comigrating and therefore difficultly interpretable polymorphic bands are, for convenience sake, included in the total number of bands alone. Segregating amplification products which were mapped on the potato genome are indicated by their locus designation. For the purpose of mapping no attempts were made to identify allelism between amplification products. Therefore, it should be realized that the AFLP markers shown on the map indicate segregating amplification products rather than genetic loci.

Table 1: Numbers and segregation types of AFLP amplification products generated with six different primer combinations.

Primer combination	Total number	Genetic model of segregating type					
E+3/M+3 ext.	of bands	1:1♀ Aa×aa	1:1♀ Aa×AA	1:2:1 Aa×Aa	1:1♂ AA×Aa	1:1♂ aa×Aa	Total
AAA/ACG	84	12	3	9	1	21	**46**
AAC/CAC	95	11	5	7	1	18	**42**
AAC/CAG	99	20	3	3	1	18	**45**
ACA/CAC	87	15	1	9	2	17	**44**
AGA/CAT	107	13	0	2	1	22	**38**
ATG/CTC	121	17	1	7	2	22	**49**
Total	593	88	13	37	8	118	**264**

Map construction: The segregation data of 138 and 163 amplification products, which were heterozygous in the female and male parent respectively, were added to the maternal and paternal data sets which contained already 150 and 146 RFLP, isozyme and morphological trait loci (Jacobs et al. 1995). The 288 markers collected in the maternal data set were grouped into maternal linkage groups. At the LOD=4 threshold level 18 linkage groups of at least four markers were identified, while a LOD=3 resulted in only eight linkage groups. Finally the correct haploid number of twelve linkage groups was established after correction for erroneous linkage relations between individual markers

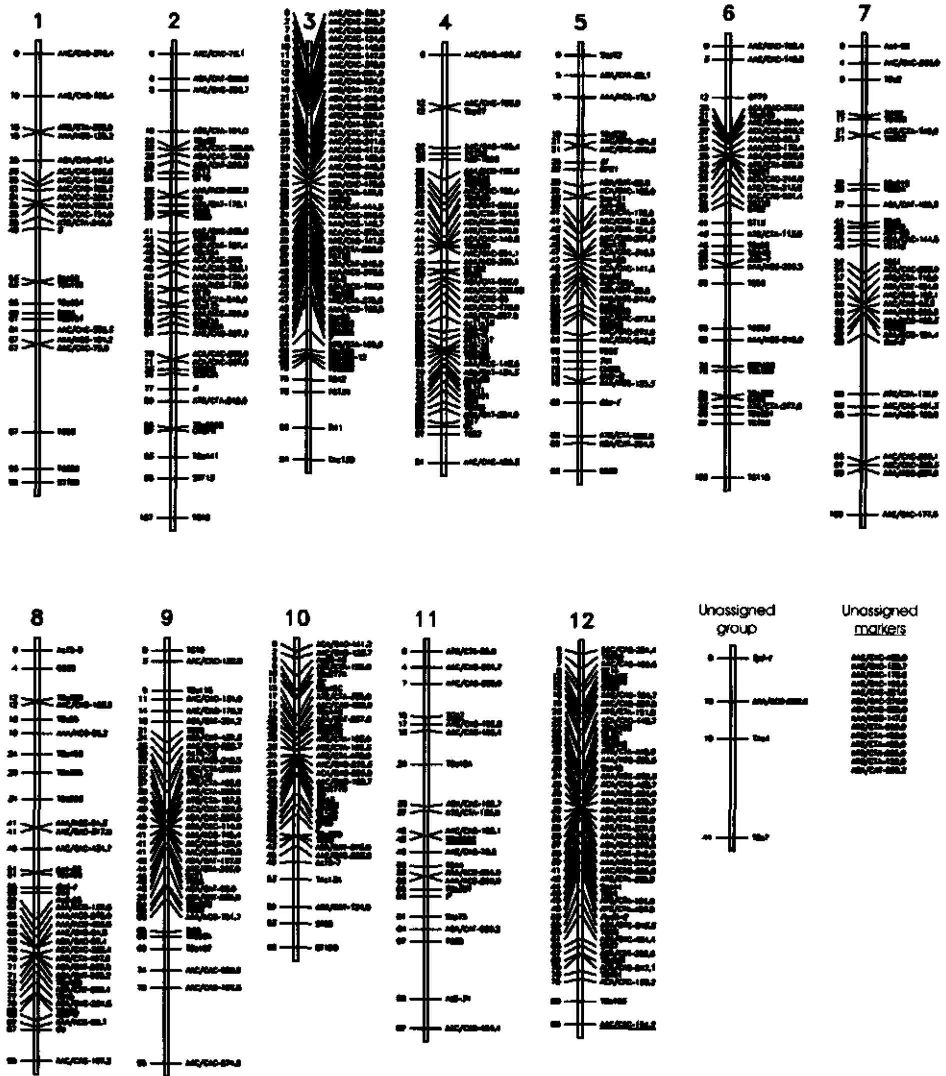

Figure 3: *Genome map of diploid potato (Solanum tuberosum L.), based on the analysis of 68 offspring genotypes. Linkage groups were established with LOD > 3.0. Individual markers mapped with a LOD < 3.0 are underlined. Chromosome numbering and orientation is according to Bonierbale et al. (1988) and Gebhardt et al. (1989).*

from different chromosomes. Chromosome-specific tomato RFLP markers (Tanksley et al. 1992) were indispensable in grouping markers. At a LOD=5 threshold level, the paternal data set, containing 309 markers, split into only ten linkage groups. RFLP markers were required to separate fused linkage groups *1 - 11* and *6 - 9*. Fifteen AFLP markers (7%) did not show linkage to any other marker (even at LOD = 2) and hence they remained unassigned.

Some maternal or paternal linkage groups were not contiguous but consisted of two unlinked pieces. Two pieces of one parental linkage group can be connected on the basis of allelic bridges (Ritter et al. 1990) to the homologous linkage group containing the loci segregating in the other parent. Low LOD gaps were found in linkage groups *1*, *7*, *8*, *11* and *12* (Jacobs et al. 1995); the LOD values of linkage between the detached pieces were 1.2, 2.6, 1.4, 2.4 and 1.3 respectively.

The localisation of AFLP markers on the genetic map of potato: Within the maternal and paternal linkage groups AFLP markers easily mapped among other marker loci. The distances and marker ordering in the resulting 24 parental linkage groups were generally in agreement with those previously found on the basis of RFLP markers alone (Jacobs et al. 1995). When a data set was used comprising only AFLP markers, again the same linkage groups were found as those found when RFLP data were included (data not shown). On the basis of allelic bridges (Ritter et al. 1990) the homologous maternal and paternal linkage groups could be merged into twelve potato chromosome maps using JOINMAP (Stam, 1993). The chromosome maps were calculated while fixing the marker orders as much as possible according to the orders already found in the separate parental linkage groups. The twelve chromosome maps shown in Figure 3 represent average maps, based on the separate maternal and homologous paternal linkage groups (not shown).

The numbers of AFLP markers assigned to each of the 24 parental linkage groups were different. For example, the maternal and homologous paternal linkage group *7* both contained nine RFLP markers, whereas 18 AFLP markers could be added to the maternal linkage map and only one AFLP mapped to paternal group. A similar situation was found for the maternal and paternal linkage group *10* accommodating three and thirteen AFLP markers, respectively. The number of AFLP markers assigned to the 24 linkage groups, relative to the number of RFLP markers per linkage group, tested by a Chi-square contingency test, was found to be significantly different (X^2_{23} = 54.5, $P < 0.001$). Likewise, the distribution of AFLP polymorphisms over maternal linkage groups was found to be significantly different from the number of AFLP polymorphisms found in the homologous paternal linkage groups (X^2_{11} = 46.9; $P < 0.001$). Within each linkage group AFLP markers mapped generally at similar regions where RFLP markers were found. However, in five out of 24 linkage groups (*3*, *6*, *7*, *9* and *12*) small groups of AFLP markers did not show recombinations. Two of these clusters of AFLP markers on linkage groups *7* and *12* contained also RFLP markers without recombination.

In six cases 1:2:1 segregating AFLP markers mapped to one parental linkage group

with high LOD scores, but no linkage (LOD < 2) was found to markers of the homologous parental linkage group. The 1:2:1 segregating AFLP marker E+ACA/M+CAC-286.8 appeared to be linked to several AFLP and RFLP markers on the maternal linkage group *2* (LOD values between 5 and 7), but without linkage to paternal linkage group *2*. Paternal linkage was detected on group *4* (LOD values between 4 and 5) without linkage to maternal group *4*. The remarkable behaviour of marker E+ACA/M+CAC-286.8 is explained as the result of two independent 1:1 segregating presence/absence polymorphisms with equally sized, and therefore co-migrating amplification products. The two overlapping 1:1 segregations from both loci were mistaken as a single marker locus having a 1:2:1 segregation. The loci are indicated on the map as E+ACA/M+CAC-286.8A and E+ACA/M+CAC-286.8B. The other 28 amplification products segregating in a 1:2:1 ratio mapped on similar positions on the homologous female and male linkage groups (two AFLPs remained unassigned).

AFLP markers with segregation ratios deviating from the expected Mendelian ratios were observed. These AFLP markers mapped to regions on chromosomes *1* and *11* where skewed segregation was found earlier in this mapping population (Jacobs et al. 1995).

The total map length increased only 6% from 1120 (Jacobs et al. 1995) to 1170 cM. For the chromosomes *1*, *3*, *4*, *7* the map length increased because of AFLP markers mapped at distal regions of the chromosome. In other regions *8* and *12* the estimated map length was reduced by internal tensity related to double cross-over events.

DISCUSSION

Identification of segregating amplification products: Using six primer combinations 227 amplification products were generated which segregated in a 1:1 ratio ($Aa \times aa$, $Aa \times AA$, $aa \times Aa$ and $AA \times Aa$; Figure 1, models 2, 4, 6 and 8), whereas only 37 amplification products segregated in the 1:2:1 ratio (Table 1). The low number of 1:2:1 segregating polymorphisms is puzzling, because in this backcross one allele of the paternal clone is identical by descent to one allele in the maternal parent. Besides, within the 1:1 segregating amplification products the number of presence/absence polymorphisms is much larger than the number of band intensity polymorphisms. The excess of 1:1 segregating presence/absence polymorphisms could be a result of the six primer combinations used. In retrospect it would have been better to choose random primer combinations, because unintentionally this selection for polymorphisms between the parental clones may have resulted in the excess of 1:1 segregating presence/absence polymorphisms. When comparing parental AFLP profiles only the $Aa \times aa$ and $aa \times Aa$ types of polymorphisms are detected, whereas the $AA \times Aa$, $Aa \times Aa$ and $Aa \times AA$ types remain undiscovered on the basis of parental profiles alone.

Inheritance of AFLP loci in the offspring of non-inbred parents: Up till now the segregation of amplification products was considered, but allelic relations between amplification products have been ignored. In a mapping population descending from non-inbred parental clones various types of segregating AFLP loci could be expected. Schematic presentations of the expected AFLP phenotypes and the underlying genetic models are shown in Figure 4. The figure illustrates that the phenotypes of the more complex AFLP loci having two or three co-dominant alleles are composed of the phenotypes of amplification products shown in Figure 1.

The AFLP assay is a multi-locus marker system which enables the simultaneous investigation of many loci. On the contrary, the (multiple) alleles of an individual locus are not necessarily amplified by a single primer combination. Putative allelic amplification products could be recognized only after linkage analysis was completed on the basis of absolute linkage found between 1:1 and 1:1, 1:1 and 1:2:1, and between 1:2:1 and 1:2:1 segregating amplification products, although complete cosegregation can not be used to prove allelism.

The inability to verify allelism between amplification products forced us to map alleles rather than loci. This leads inevitably to an overrating of the actual number of loci mapped. Examples of loci which were mapped as two or three cosegregating AFLP markers are shown in Figure 4, types A_2.1, A_2.3, A_2.4, B4, B5, and B7. The alleles of a locus as shown in Figure 4 type B1 are inevitably observed as two independent loci.

Mapping of AFLP markers: AFLP markers were mapped using a mapping population from which a detailed RFLP map was available. This resulted not only in the construction of AFLP linkage groups, but also the chromosomal origin of AFLP markers could be identified. Consequently the resulting AFLP map is in alignment with the existing potato and homoeologous tomato genome maps as published by Bonierbale et al. (1988), Gebhardt et al. (1991) and Tanksley et al. (1992).

A total of 264 AFLP amplification products was mapped relative to 193 RFLP, isozyme and morphological loci. Although AFLP mapping more than doubled the number of genetic markers, the map structure and marker orders were in general not different from the initial map. No AFLP markers were found to bridge the existing gaps in some maternal and paternal linkage groups (Jacobs et al. 1995). The small linkage group comprising the isozyme and RFLP loci TDs7 and TAc4, the isozyme locus *Tpi-1* and AFLP locus E+AAA/M+ACG-202.0 remained unassigned just like 22 previously unassigned RFLP markers (Van Eck, 1995). For four AFLP markers the inability to assign them could be attributed to the low number of observations. For the remaining ones (N=11), we have no satisfying explanation. The map length slightly increased from 1120 to 1170 cM. These results indicate that in the potato offspring studied, AFLP markers do not target other genomic regions as those already targeted by RFLPs. It appears that AFLP marker technology may substitute other marker systems.

Figure 4: Inheritance of AFLP marker loci in the progeny from non-inbred parental genotypes.

Genetic model	Mendelian Ratio	AFLP Phenotypes on autoradiogram: parents	AFLP Phenotypes on autoradiogram: offspring	Remarks
A_1. Two alleles: Allele A represents the presence and allele a the absence of an amplification product. See Fig. 1.				
A_2. Two alleles: Alleles A_1 and A_2 represent unique amplification products (A_2 migrates faster than A_1; reciprocal crosses are not depicted).				
1. $A_1A_1 \times A_1A_2 \rightarrow A_1A_1, A_1A_2$	1:1 ♂			Observed as two absolutely linked 1:1 segregating loci
2. $A_1A_1 \times A_2A_2 \rightarrow A_1A_2$	-			Not expected in a backcross
3. $A_1A_2 \times A_2A_2 \rightarrow A_1A_2, A_2A_2$	1:1 ♀			Observed as two absolutely linked 1:1 loci
4. $A_1A_2 \times A_1A_2 \rightarrow A_1A_1, A_1A_2, A_2A_2$	1:2:1			Observed as two linked 1:2:1 loci
B. Three alleles: Alleles A_1, A_2 and A_3 represent unique amplification products (A_3 migrates faster than A_2, A_2 faster than A_1). Allele a represents absence of an amplification product.				
1. $A_1a \times A_2a \rightarrow A_1A_2, A_1a, A_2a, aa$	1:1:1:1			Inevitably observed as two independant loci
2. $A_1A_1 \times A_2a \rightarrow A_1A_2, A_1a$	1:1 ♂			Not expected in a backcross
3. $A_1A_2 \times aa \rightarrow A_1a, A_2a$	1:1 ♀			Not expected in a backcross
4. $A_1A_2 \times A_1a \rightarrow A_1A_1, A_1A_2, A_1a, A_2a$	1:1:1:1			Observed as a 1:1 locus linked to a 1:2:1 locus
5. $A_1A_2 \times A_2a \rightarrow A_2A_2, A_1A_2, A_1a, A_2a$	1:1:1:1			Observed as a 1:1 locus linked to a 1:2:1 locus
6. $A_2A_2 \times A_1a \rightarrow A_1A_2, A_2a$	1:1 ♂			Not expected in a backcross
7. $A_1A_2 \times A_2A_3 \rightarrow A_1A_2, A_1A_3, A_2A_2, A_2A_3$	1:1:1:1			Observed as three linked loci
C. Four different alleles: This situation is not is not expected in a backcross				

On linkage groups *3*, *6*, *7*, *9* and *12* clustering of AFLP markers was observed. The cause of this phenomenon may be either the occurrence of multiple specific genomic sequences which are targeted by AFLP, or the occurrence of centromeric or telomeric suppression of recombination as observed in tomato (Tanksley et al. 1992). The present number of RFLP and AFLP markers relative to the genetic resolution offered by an offspring size of 68 plants, does not allow to clarify the cause of clustering of markers. Within these clusters with little or no recombination many 1:1 segregating presence/absence polymorphisms were found. As a result of the backcross nature of this mapping population, allelism could be excluded between the majority of the absolutely linked 1:1 segregating AFLPs found in these clusters (see Figure 4, type B3). This allowed us to conclude that AFLP markers E+AAC/M+CAG-582.0, E+AAC/M+CAG-446.3, E+AAC/M+CAG-285.4, E+AAC/M+CAG-241.0, E+AAC/M+CAG-142.0, E+ACA/M+CAC-470.8, E+ACA/M+CAC-261.2, E+ACA/M+CAC-315.2, E+ATG/M+CTC-293.0, E+ATG/M+CTC-261.0, E+ATG/M+CTC-220.0, E+AGA/M+CAT-444.0 and E+AGA/M+CAT-246.0 on chromosome *3*; E+ACA/M+CAC-553.6, E+AAA/M+ACG-176.4, E+ATG/M+CTC-580.0 and E+ATG/M+CTC-213.0 on chromosome *6*, E+AAC/M+CAG-93.3, E+AAC/M+CAG-192.1, E+AAA/M+ACG-509.6, E+AAA/M+ACG-492.3 and E+AGA/M+CAT-124.0 on chromosome *7*, E+AAC/M+CAG-149.0, E+ACA/M+CAC-231.5, E+ACA/M+CAC-207.5, E+ACA/M+CAC-114.0, E+AAA/M+ACG-145.4, E+ATG/M+CTC-260.0 and E+ATG/M+CTC-187.5 on chromosome *9* and E+AAC/M+CAG-286.0, E+ACA/M+CAC-438.7, E+AAA/M+ACG-575.7, E+AAA/M+ACG-395.8, E+AAA/M+ACG-284.0, E+ATG/M+CTC-307.0, E+ATG/M+CTC-191.0, E+AGA/M+CAT-346.0, E+AGA/M+CAT-266.0 and E+AGA/M+CAT-235.0 on chromosome *12*, cannot be alleles of one another.

From this it follows that with the limited number of primer combinations used in this study the greater part of the alleles were not amplified. When few putative alleles are detected AFLP polymorphisms should be based on DNA sequence variability at *Eco*RI and *Mse*I restriction sites and the selective nucleotides, rather than nucleotide insertions or deletions in template DNA.

Examples of absolutely cosegregating amplification products, which are putative multi-allelic loci, were found on chromosomes *2*, *3*, *8* and *12*: On chromosome *2*: E+ACA/M+CAC-266.0 putatively allelic with E+ACA/M+CAC-267.0; E+ACA/M+CAC-286.8*A* with E+ACA/M+CAC-165.8 or E+AGA/M+CAT-296.0); E+AAA/M+ACG-124.4 with E+AGA/M+CAT-197.4. On chromosome *3*: E+AAC/M+CAG-584.0 with E+AAC/M+CAG-582.0; E+AAC/M+CAG-446.3 with E+AAC/M+CAG-417.6; E+AAA/M+ACG-140.7 with E+AGA/M+CAT-246.0. On chromosome *8*: E+AAC/M+CAC-317.0 with E+AAC/M+CAC-434.7; E+ACA/M+CAC-87.4 with E+AAA/M+ACG-218.0 and E+AAA/M+ACG-456.6. On chromosome *12* E+AAA/M+ACG-305.6 with E+AAA/M+ACG-575.7. Genetic distances on the map larger than zero between absolutely linked markers resulted from random sampling

differences.

Allelism can also exist between two AFLP markers, one localized on the maternal and the other at a comparable position in the paternal map. Southern blot analysis with cloned bands might sort out allelism in cases where this information is needed.

Consequences of the inheritance of AFLP markers: From the various AFLP phenotypes shown in Figure 4 and observed in this mapping population it is evident that in an outbreeding species like potato the relation is unknown between the number of segregating amplification products and the number of AFLP loci. Maximizing of the amount of genetic information that can be obtained from AFLP profiles would be achieved by studying the segregation of AFLP loci, rather than the segregation of amplification products. In other words, when the different alleles which belong to a single AFLP locus can be recognized, the marker locus is genetically most informative. Especially the identification of polymorphic alleles descending from both parents (allelic bridges) are essential for the integration of the separate maternal and paternal linkage groups to one chromosome map. Only the 1:2:1 segregating AFLP markers could be used as allelic bridges but unfortunately these markers are not very informative for the estimation of linkage intensities, and represented only a minor fraction of the AFLP markers in this study. Proper integration of maternal and paternal linkage groups mainly relied upon the highly informative 1:1:1:1 segregating multiallelic RFLP markers. The disadvantage of the low fraction of 1:2:1 segregating allelic bridges is largely compensated by the large number of markers generated by the AFLP technique.

Another disadvantage connected to AFLP is the high proportion of single allele markers, which have less power to detect QTLs in non-inbred populations with non-additive intra-locus allele interactions (dominance). Proper evaluation of those QTLs and QTLs with multiple alleles is heavily depending on multi-allelic molecular markers (van Eck et al. 1994). Although multiple alleles of AFLP loci are not easily identified, two absolutely linked AFLP loci, one segregating in a 1:2:1 and the other in a 1:1 ratio can be translated easily into a multi-allelic marker with a 1:1:1:1 segregation (see Figure 4). Again this disadvantage is largely compensated by the large number of markers generated by the AFLP technique.

AFLP images: Digitized images of the AFLP profiles of the parental clones will be become available through the Solgenes Genome Database. These images will probably allow alignment of AFLP linkage groups produced by other labs to existing genome maps, provided that two assumptions are correct. First, an AFLP allele should be locus-specific. Locus-specificity is expected because the chance is very small that two independent AFLP loci in one genome have the same restriction sites, fragment size and selective bases. The second assumption is that identification of the AFLP allele is assured by accurate assessment of the mobility of the amplification product. Correct identification of

amplification products can be achieved by using an appropriate DNA size marker, such as the 10 base ladder used in this study.

A more accurate method for the identification of amplification products in other potato clones could be based on co-migration with bands present in our parental clones. In this study only one case was found where bands which co-migrated in the parents were not identical. The markers E+ACA/M+CAC-286.8A and E+ACA/M+CAC-286.8B which were mapped on non-homologous positions on linkage groups *2* and *4* were falsely interpreted as the 1:2:1 segregating amplification product from a single locus. In the corresponding situation of an RFLP probe hybridizing to another genomic sequence letters are used as well to distinguish between the loci.

Eventually, immediate localisation of traits by using bulked segregant analysis (BSA) will only be feasible when a good reference library of mapped AFLP loci is available for identification of bulk-specific AFLP alleles.

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Chapter 4

Use of allele specificity of comigrating AFLP markers to align genetic maps from different potato genotypes

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ABSTRACT

The allele specificity of AFLP markers was assessed in five relatively unrelated potato genotypes. For that, two diploid mapping populations of potato, F_1SH×RH and F_1AM×RH, were analysed reusing four and six AFLP primer combinations respectively, recently applied to analyse a genetically well-characterized backcross population BC_C×E [Van Eck et al. (1995)]. The AFLP profiles of the five parents revealed 733 AFLP markers and, under the condition that identical primer combinations were used, 131 comigrating AFLP markers were identified. After construction of five parental maps, the genomic positions of these comigrating AFLP markers were compared and 117 markers (89%) which targeted the same genomic region were assumed to be homologous. Of these putative homologues, 20 markers, each cloned from at least two genotypes, were sequenced and 19 sets of amplification products were shown to be identical. Per chromosome, the number of AFLP markers previously mapped in population BC_C×E ranged from three to eleven, which allowed a reliable assessment of chromosome numbers from individual linkage groups obtained in populations F_1SH×RH and F_1AM×RH. The high incidence of corresponding AFLP alleles was confirmed by using an additional set of five primer combinations. The 733 AFLP markers localized provide a valuable reference collection for future mapping studies in potato. As a consequence AFLPs may substitute more laborious locus specific marker techniques.

INTRODUCTION

Both for marker-assisted breeding and the isolation of genes by, *e.g.*, chromosome landing (Tanksley et al. 1994) dense linkage maps are indispensable. Molecular markers, such as restriction fragment length polymorphisms (RFLPs), randomly amplified polymorphic DNA (RAPDs), sequence characterized amplified regions (SCARs) and sequence-tagged microsatellites (STMS) allow the generation of such maps (Tanksley et al. 1989; Williams et al. 1990; Paran and Michelmore 1993; Beckmann and Soller 1988). Recently, a time and cost efficient DNA fingerprinting technique called AFLP was described by Vos et al. (1995). This technique is based on the selective, semi-quantitative PCR amplification of restriction fragments from a total genomic DNA digest. AFLP fingerprints can be tailored to produce patterns of varying complexity by changing the set of restriction enzymes used and the length and the composition of the PCR primer extensions. The AFLP technique is reliable and high numbers of molecular markers can be generated without any foreknowledge about the genetic constitution of the organism under investigation (Vos et al. (1995).

The construction of a genetic map without chromosome-specific markers results at best in a map which is comprised of a number of linkage groups corresponding to the chromosome number of the species under investigation. No chromosome numbers can be attributed to these groups and there is no means to determine the chromosomal orientation. To localize AFLP markers on the genetic map of potato, a diploid backcross population was analysed in which chromosome-specific restriction fragment length polymorphism (RFLP), isozyme and morphological trait loci were already mapped (Van Eck et al. 1995b). This backcross population is referred to as BC_C×E (Jongedijk and Ramanna 1988).

The application of molecular markers for genetic studies in crop plants relies heavily on the ability to exchange information between genetic maps obtained from different crosses. The goal of this study was to investigate whether AFLP markers can be used to align genetic maps obtained from different genotypes. Unlike other marker techniques, the AFLP assay enables the simultaneous investigation of 60 to 100 loci. This approach hinders the recognition of different allelic products amplified from a single locus. Moreover, the (multiple) alleles of an individual locus are not necessarily amplified by a single primer combination. As a consequence AFLP markers are, in contrast with RFLPs, mapped as alleles rather than loci. Therefore, the possibilities to align AFLP maps will depend on the number of alleles shared among the markers segregating in different mapping populations. In this study we analyzed the proportion of shared alleles among the polymorphic amplification products from five different potato genotypes. In addition we tested to which degree comigration of AFLP markers was due to fortuitous events rather than allelism.

Two diploid potato mapping populations, F_1SH×RH and F_1AM×RH, were analysed with primer combinations that were used before to generate AFLP markers in BC_C×E (Van Eck et al. 1995b). The parents of these mapping populations are breeding lines which

harbour introgressions of various wild *Solanum* species. Amplification products identified in different germplasms were assumed to be identical alleles if, i) they are generated with identical AFLP primer combinations, ii) show an equal electrophoretic mobility and iii) target the same genomic regions. Allelism between a number of the putatively homologous AFLP markers which conform the above mentioned criteria was tested by comparing their nucleotide sequences.

MATERIALS AND METHODS

Plant material: The mapping population coded F_1SH×RH consisted of 194 individuals. The diploid female clone SH82-93-488 originates from a cross between the diploid *S. tuberosum* clone SH76-128-1857 and a dihaploid of clone Y66-13-628 which has cultivar Amaryl, derived from *S. tuberosum* spp. *andigena* CPC1673, in its ancestry. Clone SH82-93-488 is referred to in this paper as SH. The diploid male parent RH89-039-16 originated from a cross between clone BC1034 and SUH2293. BC1034 descended from a cross between clone B (originally named USW5295.7) and clone C (originally named USW5337.3). On the basis of this pedigree information, clone C and clone RH89-039-16 have 5/16 of their alleles identical by descent. The male parent RH89-039-16 is encoded RH.

Mapping population F_1AM×RH comprised 102 F_1 genotypes derived from the diploid cross 3778-16 × RH 89-039-16. The dihaploid female parent 3778-16 was derived from the tetraploid breeding clone AM78-3778 which is an interspecific hybrid between *S. tuberosum* and several wild *Solanum* species including *S. tuberosum* spp. *andigena, S. vernei, S. vernei* ssp. *ballsii* and *S. oplocense*. Clone 3778-16 is referred to in this paper as clone AM. Clone RH was used as the male parent to generate population F_1AM×RH.

AFLP protocol: DNA isolation was performed on frozen leaf tissue (Van der Beek et al. 1992) or small tuber parts. The AFLP method is extensively described by Vos et al. (1995). Template DNA was prepared using the restriction enzymes *Eco*RI and *Mse*I. Amplification of the restriction fragments was performed using two sets of primers. Primers within set E all include the sequence 5'-GAC TGC GTA CCA ATT C, set M primers have the sequence 5'-GAT GAG TCC TGA GTA A in common. The code following E or M refers to additional selective nucleotides at the 3'-end of the primer (*e.g.* primer E+AAA refers to a primer with selective nucleotides AAA added to the 3'-end of the core *Eco*RI-primer).

The primer combinations E+A/M+A and E+A/M+C were used for pre-amplification of primary template. Six selective nucleotides were used to generate AFLP fragments from secondary template. The nucleotide sequences of the primer combinations are listed in Table 1. Amplification reaction products generated from population F_1AM×RH were loaded on 5 % polyacrylamide gels (Vos et al. 1995). Slightly modified electrophoresis conditions were applied to analyse the amplification products generated from population

Table 1. The number of markers generated by AFLP analysis of three parental clones using twelve primer combinations. The primer combinations designated in italics were also used to analyse the reference population BC_C×E.

Primer combination[a]	Total no. of bands	Number of AFLP markers identified per parental clone					
E+3/M+3		**C**	**E**	**SH**	**AM**	**RH**	**Total[b]**
AAA/ACG	102	24	29	33	19	32	90
AAC/CAC	114	20	24	34	32	34	100
AAC/CAG	99	24	21	29	23	37	94
ACA/CAC	82	24	27		12	16	58
AGA/CAT	117	12	22		33	23	70
ATG/CTA	166	23	26	46	43	58	138
AAA/ACC	165				24	21	44
AAC/CCA	80				16	17	28
AAC/CTG	70				13	16	27
ACA/CCT	60				14	5	16
ACA/CGT	29				10	8	16
ATG/CTC	128			37		22	52
Total	1212[c]	127	149	179	239	289	733

[a] The primer combinations designated in italics were also used to analyse the reference population BC-_C×E.

b For calculation of the total number of AFLP markers identified, putatively homologous AFLP markers (among others markers which exhibited an $Aa \times Aa$ segregation type) were included only once.

c The total number of amplification products exceeds the number which should be expected on the basis of the heterozygosity percentages of the parents. Approximately 15% of the fragments which were heterozygous in either of the parents could not be scored unambiguously in the progeny. These fragments were not regarded as an AFLP marker though they were included in the heterozygosity percentages.

F_1SH×RH. Amplification products were loaded on 4.5% polyacrylamide gels using 1 × TBE (100 mM Tris, 100 mM boric acid, 2 mM EDTA) at the cathodal site, and 0.5 M NaAc in 1 × TBE at the anodal side of the gel. These modified electrophoresis conditions resulted in an ionic gradient and increased the number of AFLP fragments identified. Amplification products were visualized by autoradiography. Nomenclature of AFLP markers was based on the primer extension and the electrophoretic mobility. The mobility of the amplification product was determined by comparison with a SequaMark 10 bp-ladder (Research Genetics, Huntsville AL USA).

Using a particular primer combination, a subset of the amplification products generated from the offspring of the crosses F_1SH×RH and/or F_1AM×RH have the same mobilities as AFLP markers mapped in BC_C×E and were indicated by identical names. Segregating amplification products generated from two or more genotypes which are characterized by equal primer extensions and mobilities are mentioned throughout the text as comigrating AFLP markers.

Data analyses : For data analyses three types of segregation patterns of AFLP products were recognized:

(1) In case an amplification product was found in one parental clone only and segregated as a presence/absence polymorphism in the offspring, the underlying genetic model is supposed to be $Aa \times aa$ or $aa \times Aa$. Maternally and paternally segregating presence/absence polymorphisms are presented in Figure 1.
(2) In case amplification products were found in both parental clones at identical positions in the gel but with different band intensities and in which the major band is of double intensity as compared to the minor band, the underlying genetic model is supposed to be $Aa \times AA$ or $AA \times Aa$. In Figure 1, an example of a paternally (clone RH) segregating band intensity polymorphism is shown.
(3) In case both types of polymorphisms (presence/absence polymorphisms and band intensity polymorphisms) were observed among the offspring phenotypes descending from parents with the weaker band intensity phenotype, the underlying genetic model is supposed to be $Aa \times Aa$. An example is shown in Figure 1.

The zygosity of the offspring genotypes was independently determined by two persons. The three segregation types were converted to mapping data by giving them the appropriate JoinMap code (Van Eck et al. 1995b).

Map construction: AFLP data of both F1 populations were analysed separately according to their parental origin as described by Eck et al. (1995b) and Jacobs et al. (1995) using the software package JoinMap 1.4 (Stam 1993). To create linkage groups, LOD scores were stepwise decreased from 8.0 to 3.0. Linkage groups of the SH, AM and RH map were discriminated at LOD = 7.0, 5.0 and 8.0 threshold levels respectively. At lower LOD scores some linkage groups agglomerated due to skewed segregating markers. Erroneous linkage between AFLP markers was recognized via a chi-square contingency test using the program Linkage-1 (Suiter et al. 1983). In case the number of linkage groups differed from the correct haploid number of twelve linkage groups, the grouping of comigrating AFLP markers was compared with the grouping in the combined BC_C×E map. A set of true AFLP alleles localized on a single linkage group of the map of clone SH, RH or AM, should be localized on one of the chromosomes of the BC_C×E map. Fused linkage groups could be separated when two sets of comigrating AFLP markers were localized on a single linkage group in clone SH, AM or RH but on different chromosomes in the BC_C×E map.

Map distances were calculated using Kosambi's mapping function. The datasets were inspected for the occurrence of "singletons", *i.e.* parental scores which are flanked by alternative scores of the other parent (Säll and Nilsson 1994). Singletons, which suggest a frequent cross-over event, were reinspected on autoradiograms and, if necessary, rectified.

Assignment of chromosome numbers: Chromosome numbers were assigned to linkage groups of clone SH, AM and RH based on the alignment with at least three putatively homologous AFLP markers on the BC_C×E map. Two AFLP markers characterized by

equal primer extensions and mobilities were assumed to be putatively homologous when they target the same genomic region in different genotypes. Alignment of linkage groups was not necessarily restricted to AFLP markers which putative homologues were identified previously in clones C and E. Alignment of a linkage group in *e.g.* clone SH with only one putatively homologous BC_C×E marker could also be achieved via AFLP markers putatively homologous to AM and/or RH markers. These AM and/or RH markers could be linked to markers which were, in their part, putatively homologous to C and E markers.

When ordering differences were encountered between the parental maps, alternative marker orders were submitted to JoinMap using the option "fixed orders". The alternative marker order was accepted when it resulted in a decrease in the chi-square value for mapping. Maps were drawn with the aid of the graphical package DrawMap (Van Ooijen 1994a) in orientations homologous to the potato map of Gebhardt et al. (1991) and Tanksley et al. (1992).

Distribution of markers along the map: A chi-square test for goodness of fit was used to determine if the AFLP markers were randomly distributed within a linkage group. If the distribution of markers within a linkage group is completely random, the probability p_i to find a given number of markers is equal for every interval at that linkage group. For n_i being the observed number of markers for each interval i and n being the total number of markers for each linkage group the test criterion is

$$X^2 = \sum_{i=1}^{k} \frac{(n_i \quad np_i)^2}{np_i}$$

where k is the number of intervals per linkage group. The test criterion approximate a chi-square distribution when the expected number of markers per interval np_i is set to five (Cochran 1954). Since n is known, the probability p_i can be calculated and the interval size per linkage group follows from the product between p_i and the length of that particular linkage group (in cM). The null hypothesis for a random distribution is rejected when the test criterion is greater than the $X^2_{k-1\,[.05]}$-value.

When markers were not equally distributed, the linkage group was inspected for regions with a high marker density. The linkage group was divided in overlapping segments with the calculated interval size starting from the first marker followed by every subsequent marker. The number of markers within a segment was tested for deviation from the expected number of five as described above. A cluster was defined as a genomic region containing segments with separate test criterion values X^2_i which exceed the $X^2_{1\,[.05]}$-value.

Sequencing of AFLP markers: A subset of putatively homologous AFLP markers originating from different genomic locations was sequenced. Only markers which were

clearly separated on autoradiogram were selected. Apart from four exceptions, markers identified in one of the related clones C, E and RH were compared with the unrelated clones SH and/or AM.

In order to sequence an amplification product, the band was cut out of the polyacrylamide gel, transferred to an Eppendorf tube, overlaid with 100 µl bidest and incubated for 20 min at room temperature. Subsequently, the supernatant was discarded, 100 µl bidest was added and the content was mixed using a pipet tip. The solution was incubated for 15 min at 65°C and left overnight at room temperature. Debris in the solution was spun down and the supernatant was transferred to a fresh tube. An aliquot of 5 - 7.5 µl was re-amplified using the appropriate selective primer combination and the AFLP-PCR temperature profile (Vos et al. 1995). The amplification product was loaded on a 3 % Metaphor (FMC, Rockland ME, USA) agarose gel in TAE buffer (Sambrook et al. 1989). The DNA band was excised from the agarose gel and purified using the GlassMax Isolation Matrix System (Gibco BRL, Gaithersburg MD, USA). When the AFLP fragment was smaller than 150 bp, the re-amplified fragment was purified using the QIAquick PCR purification kit (Qiagen Inc. Chatsworth, USA). The AFLP fragment was cloned into pGEM-T (Promega, Madison WI, USA) according to the recommendations of the manufacturer, using a vector:insert ratio of 1:3. Competent DH5a-cells (Gibco BRL) were transformed with 1 µl ligation mix and recombinant clones were identified by blue/white selection. The sequences of two positive colonies were analysed.

DNA sequence analysis of cloned fragments was carried out using an automated fluorescent DNA sequencer (A.L.F. Pharmacia LKB, Uppsala, Sweden). The sequence reaction was based on the dideoxy method of Sanger et al. (1982) and performed using a PCR sequence kit (Amersham, Little Chalfont, UK) with fluorescently labelled M13 sequencing primers. The DNA sequences were compared using the GCG computer package (Genetics Computer Group, WI, USA). The number of internal sequence differences between AFLP markers were corrected for the sequencing error rate. The sequencing error rate was determined on plasmid vector sequences of comparable lengths.

In a few instances non-identical insert sequences were obtained from a single parent. The analyses of two extra positive colonies from this parent sufficed to identify the marker sequences homologous to the sequences derived from a second parent.

RESULTS

Identification of AFLP markers and construction of parental maps: Population F_1SH×RH was analysed using five primer combinations (Table 1). Out of the 303 segregating amplification products, 179 AFLP markers segregated from the maternal parent SH and 177 from the paternal parent RH. These markers were used for the construction of parental maps. The SH map consisted of 12 linkage groups with a total map length of 884 cM. Using these primer combinations, three markers segregating from clone SH and seven RH markers remained unassigned (at LOD = 3.0).

Eleven primer combinations were used to analyse population F_1AM×RH (Table 1). A total of 419 AFLP markers segregated in this population. The map of the maternal clone AM consisted of 235 markers in 12 linkage groups with a total map length of 735 cM. Four markers remained unassigned on the AM map and 223 markers were used for construction of the RH map.

The RH map is based on the analysis of both population F_1SH×RH and F_1AM×RH. All the RH markers identified by analysing population F_1AM×RH were recovered in population F_1SH×RH using the same four primercombinations. Nevertheless, the segregation type of a marker may hamper its identification in a particular population; presence/absence polymorphisms are scored more reliably as compared to intensity polymorphisms. In total 289 AFLP markers were identified of which 285 could be located on the map. Four markers remained unassigned. The RH map comprised 13 linkage groups encompassing 857 cM.

The total number of AFLP markers amplified per primer combination for each of three parental clones is presented in Table 1. Heterozygosity indicia for clones SH, AM and RH, estimated by dividing the number of heterozygous bands by the total number of bands, are 63%, 70% and 73%, respectively.

Alignment of the parental maps with the map of population BC_C×E: For the analysis of clones SH, AM and RH, primer combinations previously used to generate markers in BC_C×E were examinated again. This gave rise to respectively 33, 38 and 60 AFLP markers which comigrated with markers previously mapped in population BC_C×E. Linkage analyses revealed that the majority, 29, 34 and 54 AFLP markers, showed a similar grouping as compared to the grouping found in the BC_C×E map. The 117 putatively homologous markers were used to align the SH, AM and RH maps with the BC_C×E map.

For all three parental clones, these putatively homologous markers were distributed over the twelve chromosomes. The difference between the number of potato chromosomes and the number of linkage groups found in clone RH could be ascribed to two unconnected parts of chromosome *7*.

Presumably, most of the 14 markers which resided at non-corresponding linkage groups were non-homologous. In seven cases, slight mobility differences were observed between AFLP markers which mobilities were initially judged to be equal. These markers were renamed. The molecular weights of the remaining seven sets of putatively homologous AFLP markers were indistinguishable. These markers were discriminated by adding suffix "A" and "B" to the marker names.

Besides unidirectional comparison of the obtained AFLP linkage groups with the grouping observed in BC_C×E, the grouping of potentially homologous AFLP markers identified in clone AM, RH and SH were compared mutually. In Table 2, the total number of putatively homologous AFLP markers is presented which were identified between the five parental potato genotypes. No correlation was observed between the relatedness of the parental genotypes and the number of putatively homologous AFLP markers identified

between clones. This finding agrees with the small number of *Aa* × *AA* and *AA* × *Aa* segregating AFLP markers found in the backcross population BC_C×E (Van Eck et al. 1995b). These data support previous work indicating that co-ancestry between diploid clones will not lead to increased levels of homozygosity (Gebhardt et al. 1989).

Table 2. Number of putatively homologous AFLP markers between the different parental clones generated with six primer combinations[1].

Parental clones	SH	AM	RH	E	C
AM	23[b]	-			
RH	50[b]	55	-		
E[a]	14[b]	16	35	-	
C[a]	33[b]	31	52	34[c]	-

[a] The 5/16th of the fraction of alleles between clone C and RH and half of the fraction of alleles between clone C and E are identical by descent.

[b] Four primer combinations and different electrophoresis conditions were applied to analyse population F_1SH×RH.

[c] This figure differs slightly from Van Eck et al. (1995).

A part of an autoradiogram is shown in Figure 1. Primer combination E+AAC/M+CAG gave rise to 42 AFLP markers in BC_C×E. Amplification products generated from the three parental genotypes SH, AM and RH as well as from clone C and E are loaded next to each other for direct comparison of marker mobilities. Comigrating AFLP markers identified in clone SH and clone RH, such as AAC/CAG-143.6 and AAC/CAG-123.7 (Figure 1), segregated according to the *Aa* × *Aa* type.

Comparison of marker ordering between the different maps: Similar marker orderings of putatively homologous AFLP markers were generally observed when the parental maps of the homeologous chromosomes were compared. An example is shown in Figure 2a, in which the maps of chromosome *11* are shown. Additionally, the combined BC_C×E map of this chromosome is depicted (Van Eck et al. 1995b). Small order differences among putatively homologous AFLP markers were observed mostly in the central parts of the linkage groups, whereas marker orders on the distal parts of linkage groups generally agreed with each other.

An example of differences in marker order between the three parental maps is shown for chromosome *7* (Figure 2b). The putatively homologous marker ATG/CTA-140.0 is located between markers ATG/CTA-347.0 and AAC/CAC-110.8 at the map of clone AM, whereas marker ATG/CTA-140.0 remains on the unconnected part of the chromosome *7* map of clone RH. At the other end of chromosome *7* of clone SH, the distance between markers AAC/CAC-184.8 and AAC/CAC-177.6 is 20 cM, while a 10 cM distance and an opposite order was found on the map of clone RH.

The difference of map lengths between the SH map at the one hand and the AM and RH maps at the other hand is significant (at $P < 0.001$) when a paired *t*-test was applied on

the difference in map lengths of the sets of corresponding chromosomes. This may be due to (1) the more interspecific background (Gebhardt et al. 1991) of clone AM as compared to clone SH and (2) a reduction of recombination frequency in the male meiosis of clone RH as compared to the female meiosis of clone SH as was found in solanaceous mapping studies (De Vincente and Tanksley 1991, Gebhardt et al. 1991).

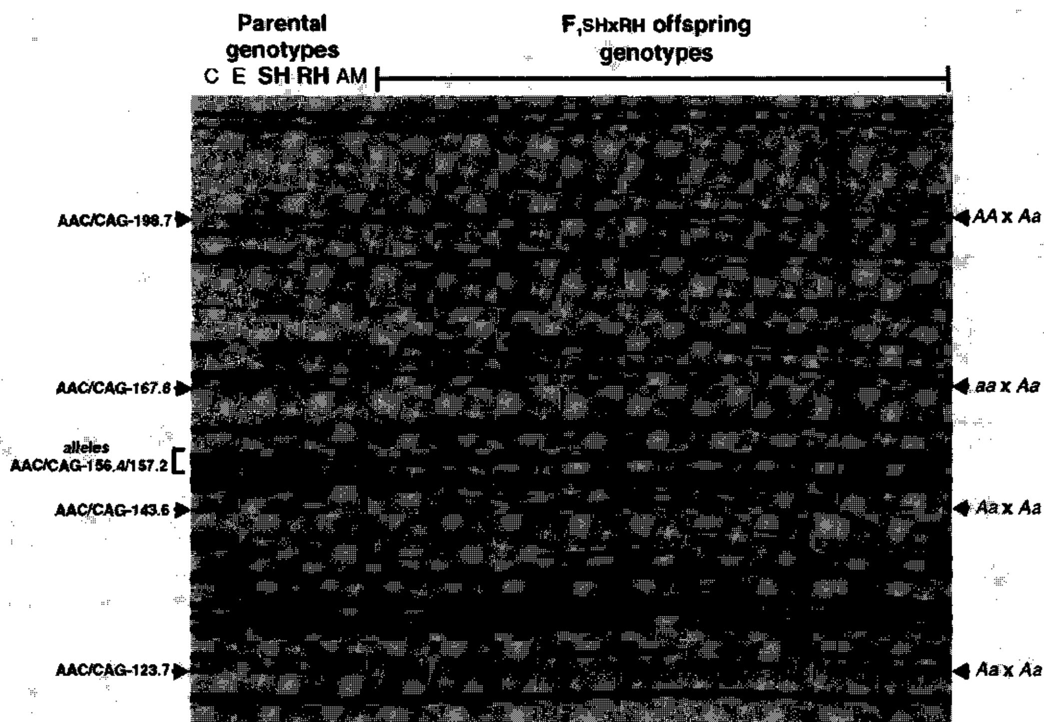

Figure 1: *AFLP profile of 16 progeny lines of population F_1SH×RH, generated using primer combination E+AAC/M+CAG The parents of population BC_C×E, F_1SH×RH and F_1AM×RH are indicated "C", "E", "SH" "AM" and "RH" respectively. A number of comigrating AFLP markers are indicated at the left. Genetic models of different segregation types of these markers are indicated at the right. Alleles AAC/CAG-156.4/157.2 belong to a single locus.*

The distribution of markers on the parental maps: A non-random distribution of AFLP markers was observed in the three maps. Clustering of AFLP markers was found at chromosomes *1*, *4* and *7* of clones SH, RH and AM, respectively. A chi-square test for goodness of fit showed that these chromosomes contained an interval at which the number of markers found was significantly higher than expected under assumption of a random distribution (at $P \geq 0.05$). Presence of putatively homologous markers within these intervals indicated that these clusters represent similar genomic regions. Figure 2b shows a subset of putatively homologous AFLP markers which are clustered on the chromosome *7* maps of the five genotypes. The cluster at chromosome *1* of clone RH was most extreme ($X^2 = 156.8$; $P < 0.0001$); a total of 34 markers mapped within an interval of 10 cM. In addition, clusters were found at chromosome *5* of clone RH and AM, chromosome *3* of clone AM and chromosome *12* of clone SH. In clone RH, a large gap, devoid of any genetic marker was found at chromosome *7*.

DNA sequence analysis of putatively homologous AFLP markers: Evidence for allelism of putatively homologous AFLP markers was obtained by DNA sequence comparison of a subset (16%) of the total number of putatively homologous AFLP markers. The results of pairwise comparisons between DNA sequences listed in Table 3, showed sequence identity of 19 out of 20 AFLP markers (Table 3). The single exception, fragments ATG/CTC-213.0 identified in clone RH and E showed an identical mobility on gel. These markers resided at similar positions on the same chromosome. Nevertheless, four replicates taken from the two parents showed that DNA sequences were different.

AFLP markers AAC/CAC-131.9/133.6 mapped on chromosome *9* and markers AAC/CAG-156.4/157.2 mapped on chromosome *11* both consisted of two DNA bands at nearby positions at autoradiogram. In both instances, the bands perfectly alternated at autoradiograms of the three mapping populations (a total of 364 genotypes). Sequence analysis revealed that the difference for both pairs of bands was caused by a deletion of one nucleotide and a single nucleotide substitution. It is concluded that each pair of bands contained two AFLP alleles of the same genetic locus. It is noted that in total 10 loci are identified in which two AFLP markers are produced with the same primer combination, are in repulsion phase and show a recombination frequency of 0.0.

Occasionally, different AFLP fragments were cloned from a sample which should contain only one AFLP marker. Therefore, sequence identity was required between at least two AFLP markers per dissected band before it was decided that the respective marker sequence was obtained. The non-homologous DNA fragments, which always contained the E-primer and the M-primer sequences, are possibly minor accessorial products resulting from less specific annealing temperatures at the final stages of the touch down PCR profile.

The results of the pairwise comparisons indicate that the majority of the other, non-sequenced putative homologues, as identified in this paper, will be identical alleles.

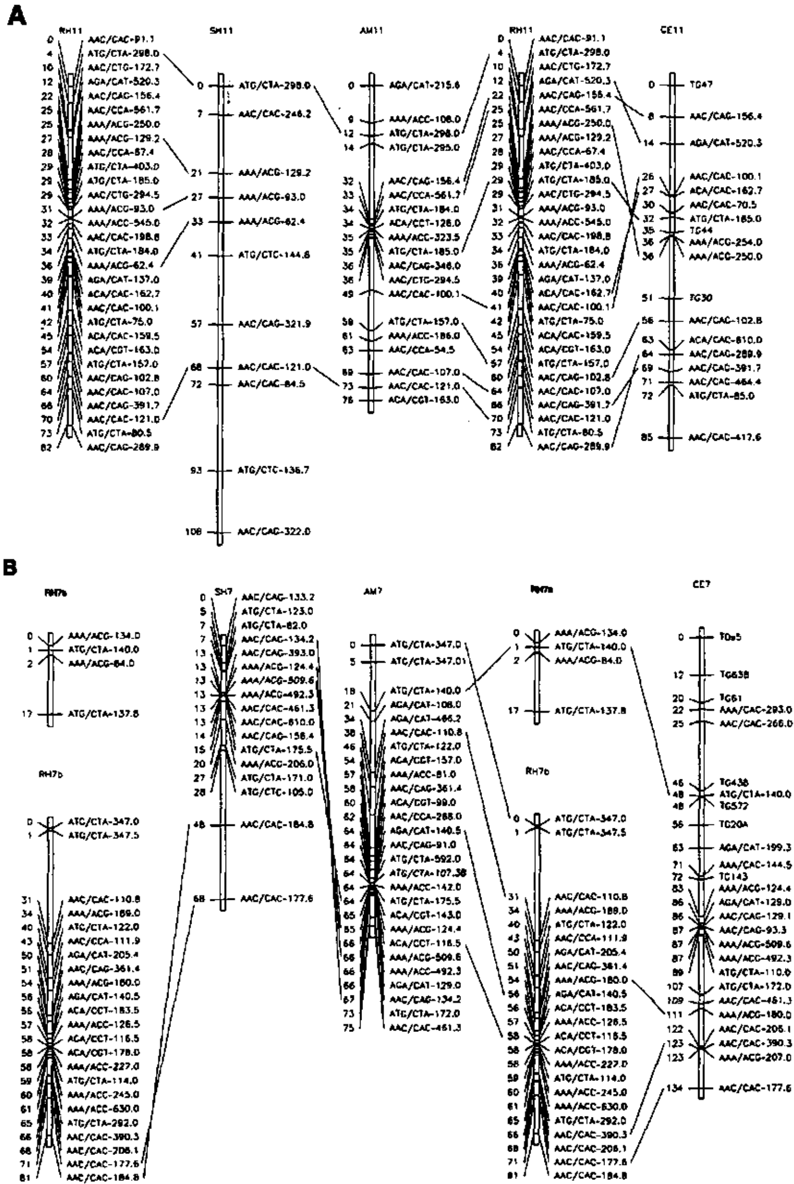

***Figure** 2: Comparisons between maps of clones SH, AM, RH and the combined BC_C×E map of chromosome* 11 *(presented in A) and chromosome* 7 *(presented in B). RFLP markers which were previously used to assign chromosome numbers in population BC_C×E are indicated with a TG or TDs prefix (Van Eck et al., 1995). Putatively homologous AFLP markers which were used to align the maps are connected with a line.*

Table 3: Results of sequence comparisons between putatively homologous AFLP markers. The names, sizes and positions of absolutely linked AFLP markers are given which were allelic on the basis of sequence information.

AFLP allele[a] E+3/M+3	Size[b] (bases)	Parental origin	Chromosome number	Number of variable internal nucleotides
AAC/CAG-236.0	233	SH, C	2	4
AAA/ACG-163.5	163	AM, E	3	3
AAA/ACG-336.7	333	AM, RH	4	0
AAC/CAC-377.6	375	SH, C	5	3
AAA/ACG-178.7	177	RH, E	5	0
ATG/CTC-213.A	210	RH	6	Not homologous to E; initially
ATG/CTC-213.B	210	E	6	identified as ATG/CTC-213
AAA/ACG-509.6	509	SH, AM, C	7	5
AAA/ACG-124.4	121	SH, AM	7	0
AAC/CAC-184.8	181	SH, RH	7	1
AAC/CAC-177.6	176	SH, RH, C	7	5
AAA/ACG-456.6	459	SH, C, E	8	10
AAA/ACG-137.6	136	SH, C, E	8	0
AAA/ACG-242.5	239	AM, C	9	Not homologous within a stretch of 46 nucleotides
ATG/CTC-262.0	260	AM, RH	9	5
AAC/CAC-132.8	131	RH	9	2: AAC/CAG-132.8 and AAC/CAG-131.9 are alleles of
AAC/CAC-131.9	130	AM, RH, C, E	9	one locus[c]
ATG/CTC-158.0	155	RH, E	10	1
AAA/ACG-136.6	132	SH, RH	11	0
AAC/CAG-157.2	154	AM, C, E	11	2: AAC/CAG-157.2 and AAC/CAG-156.4 are alleles of
AAC/CAG-156.4	153	AM, RH, C, E	11	one locus[c]

[a] Size estimated from autoradiogram relative to a 10 base ladder.

[b] Size based on sequence analysis. The length of the AFLP marker include the primersequences.

[c] See text.

DISCUSSION

In potato AFLP markers can be used to identify linkage groups in genetically uncharacterized genotypes. Reuse of primers sets, that were previously used to superimpose a large set of AFLP markers on a well-defined potato map (Van Eck et al. 1995b), resulted in numerous comigrating AFLP markers. The vast majority of these comigrating AFLP markers was shown to be allelic.

The criterium for the selection of potentially useful markers was comigration given that an identical primer combination was used. Identical nucleotide composition on the extremities of markers and similar mobilities apparently give little information about the core DNA sequences of these markers. However, when positions of potentially allelic markers on the maps are compared, 89% is localized on similar map positions. The

remaining 14 markers were localized on different map positions. Since close re-examination of the autoradiograms showed subtle mobility differences for 7 out of 14 markers, these markers are presumably non-homologous. The other half may in part be explained by structural differences between the genomes analysed. The underlying assumption for homology between comigrating AFLP alleles is violated when homologous segments are present on non-homologous chromosomes. Indications for differences in genome synteny are provided by comparing RFLP maps of potato species (Brown et al. 1995) as well as tomato species (Van Ooijen et al. 1994b).

Twenty out of 117 putatively homologous AFLP markers were selected and sequenced. Nineteen putatively homologous markers were shown to be identical. Hence, primer extension and mobility are highly informative provided that mobilities are determined very accurately. At least two factors contributed to this: (1) AFLP markers are amplified under very stringent reaction conditions. It is unlikely that two amplification products of identical size arise due to mismatches in primer-template annealing during PCR. (2) On polyacrylamide gels, the mobility of an amplification product can be estimated very accurately, mostly in tenths of nucleotides. Therefore, small differences, not only in size but also in nucleotide composition will change the mobility of a marker in a polyacrylamide gel. Given the average number of 101 fragments identified per AFLP reaction ranging in size from 50 to 500 nucleotides and an approximate number of 3500 different band positions per gel we estimate that per assay the probability of coincident comigration is 0.03 (101/3500).

Based on the putative homology of comigrating AFLP markers, the three parental maps were aligned with the existing potato map. As a rule of thumb, we used at least three putatively homologous markers per linkage group for chromosome identification. The rationale for using three markers is that if one marker is misidentified, the probability for correct chromosome assignment is still $P = 0.99$ ($1-(0.11 \times 0.11)$). Overall, the order of putatively homologous AFLP markers along the different maps agreed with each other although genetic distances differed markedly in certain intervals. The observed differences between the maps were in most cases the result of random sampling differences between the parental data sets. It is noted that the genomic position of putatively homologous AFLP markers on either genetic map was calculated on the basis of recombination values observed in different parental meioses. In addition, examining the raw datasets revealed that missing data were often found at regions with ambiguous marker orders. The fraction of missing data points for population F_1AM×RH, the largest single population dataset, was 15.4%. The genotyping error rate obtained after rescoring autoradiograms at positions where singletons were encountered was estimated to be $\leq$ 0.01%. Finally, putatively homologous AFLP markers were often scored as 1:2:1 segregating markers. These markers have the $Aa \times Aa$ segregation type of which only the AA and aa offspring classes are informative. The 50% reduction of the mapping information may result in less accurate orderings of the 1:2:1 segregating markers relative to 1:1 segregating markers. This may also serve as an explanation for the observation that 14 out of 19 E+ATG/M+CTC markers segregating in a 1:2:1 ratio remained unassigned on the SH map.

A non-random distribution of AFLP markers was found on the three maps, mainly due to the occurrence of regions in which the markers are clustered. This clustering may be due to reduced recombination rates caused by the presence of unrelated genetic material in one of the parents. Recombination is known to be suppressed in regions in which the genotype is heterozygous for chromosomal segments originating from distantly related species (Rick 1969). However, eleven clusters were found on corresponding regions of the parents and in only two genomic regions the observed clustering was confined to one parental genotype. Hence, the most likely explanation for the observed clustering is a fixed localization of recombination suppression in Solanaceous genomes, *i.e.* centromeric and heterochromatic regions (Tanksley et al. 1992; Sherman and Stack 1995).

A large gap lacking any detectable marker was observed at chromosome *7* of clone RH. Gaps in a map may result from high frequencies of recombination in that particular region (Kesseli et al. 1994; Becker et al. 1995). In addition, the identification of AFLP markers in this study may be biased to more AT-rich regions because *EcoRI* (G↓AATTC) and *MseI* (T↓TAA) were used for a total digestion of the genome.

When the separate parental maps were compared with the combined BC_C×E map, substantial differences in marker orders were observed. The mapping algorithm applied assumes that recombination distances are homogeneous when maps are merged (Stam 1993). However, the non-random marker distribution implicates that this assumption is violated. In addition, re-orderings in combined maps often result from biased estimates of recombination frequencies when datasets are lumped (Van Eck 1995a). This source of map differences should be reckoned with if marker orders between combined and single genotypes maps are compared.

The use of AFLP markers for alignment of linkage groups within Solanaceous crops seems to be confined to the species level and not to the genus level. Comparison of AFLP patterns of potato with profiles generated from tomato (*Lycopersicon esculentum*) and pepper (*Capsicum annuum*) shows that these species have hardly any AFLP marker in common (data not shown). Probably, RFLPs are more appropriate if one aims at relating more distantly related genomes. (Bonierbale et al. 1988; Tanksley et al. 1988; Hulbert et al. 1990; Whitkus et al. 1992; Ahn and Tanksley 1993).

Allelism between putatively homologous AFLP markers could be applied to study genealogical relationships. Phylogenetic inferences based on electrophoretic studies assume homologies between molecular markers with similar mobilities (Swofford and Olsen 1990). In this paper, we showed that for potato 89% of the AFLP markers, characterized by primer combination and mobility only, are indeed allelic. This finding, together with the abundance of AFLP markers makes the technique very useful for phylogenetic studies at the species level.

The maps of the three genotypes analysed together with the two parental clones of population BC_C×E contain information on the genomic positions of 733 different AFLP markers. This reference collection facilitates future genetic studies and enables the interpretation of AFLP linkage maps from other potato genotypes. For some applications, AFLPs may substitute more laborious marker techniques. As demonstrated in this report

numerous shared alleles can be identified among AFLP markers segregating in different mapping populations. With this information we estimate that only three to five primer combinations are required to determine the chromosomal region where a gene of interest is localized. Detailed information on the positions of the 733 AFLP markers as well as DNA template of the five parental clones is available upon request.

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Chapter 5

An online catalogue of AFLP markers covering the potato genome

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ABSTRACT

An AFLP marker catalogue is presented for gene mapping within cultivated potato. The catalogue is comprised of AFLP fingerprint images of 733 chromosome-specific AFLP markers which are mapped relative to 220 RFLP loci, isozyme loci, morphological characteristics and disease resistance traits. Use of the catalogue is based on identification of common AFLP markers which are visually recognized on autoradiogram images as co-migrating bands in fingerprints generated from different genotypes. Images of AFLP fingerprints combined with detailed information on the genomic location of all AFLP markers are available at URL: http://www.spg.wau.nl/pv/aflp/catalog.htm. It is demonstrated that the comparison of autoradiogram images and subsequent identification of common AFLP markers solely are efficient means for alignment of linkage groups and mapping target genes.

Abbreviation: URL: uniform resource locator.

INTRODUCTION

Efficient applications of molecular markers in crop plants, *e.g.* gene mapping, marker assisted selection and genotyping, rely heavily on the ability to exchange information between genetic maps obtained from different crosses. At present, restriction fragment length polymorphisms (RFLPs) are predominantly used as anchor points for map comparison. The locus-specificity of RFLP markers is based on DNA-DNA hybridisation and allows the prediction of linkage relationships between loci identified in different species (Tanksley et al. 1989). However, RFLPs are tedious to handle and the number of genotypes which can be screened in a short time span is limited. Alternatively, sequencing of RFLP probes allows the design of specific primers which can be applied to convert the RFLP marker into a PCR based marker. The locus-specificity of such markers is generally maintained and resides in the specific priming of two oligonucleotides to the template DNA before PCR amplification occurs. PCR based markers are relatively easy to perform but genetic changes that are detectable by RFLP analysis may not be identified. An additional drawback may be that the use of specific oligonucleotides resolve only one locus per assay.

The recently developed AFLP marker technique (Vos et al. 1995) is unprecedented with respect to the efficiency in the number of markers generated (Büschges et al. 1997; Simons et al. 1997; Thomas et al. 1995). AFLP allows the simultaneous identification of up to 50 loci per assay. For potato a number of AFLP maps have been developed which are aligned with the genetic map of potato (Gebhardt et al. 1991; Tanksley et al. 1992; Van Eck et al. 1995b) and comprise of over 700 markers (Rouppe van der Voort et al. 1997). In addition, the locus-specificity of AFLP was substantiated by sequencing AFLP markers which had the same electrophoretic mobility and targeted equivalent map positions in different genotypes.

To provide access to the information on potato AFLP markers, a catalogue is presented comprising of 733 AFLP markers mapped. Exchange of AFLP marker information via this catalogue is based on visual comparison of electrophoretic mobilities of the AFLP fragments with markers in newly generated AFLP-images from a potato genotype of interest. The catalogue is readily available from the World Wide Web and offers the opportunity to use molecular marker information without the need of sending probes, oligonucleotides or sequence information. This report describes 1) the contents of the catalogue, 2) the feasibility of the catalogue for comparative mapping and 3) its current and future applications.

The catalogue

In the catalogue, images of AFLP fingerprints are shown which are generated from five diploid *Solanum tuberosum* genotypes (Table 1). The catalogue contains information on the genomic position of 733 AFLP markers. These AFLP markers are localised on the genetic map of potato by means of 197 RFLP loci (Van Eck et al. 1995b). The primer combinations used and the numbers of markers scored per primer combination are given

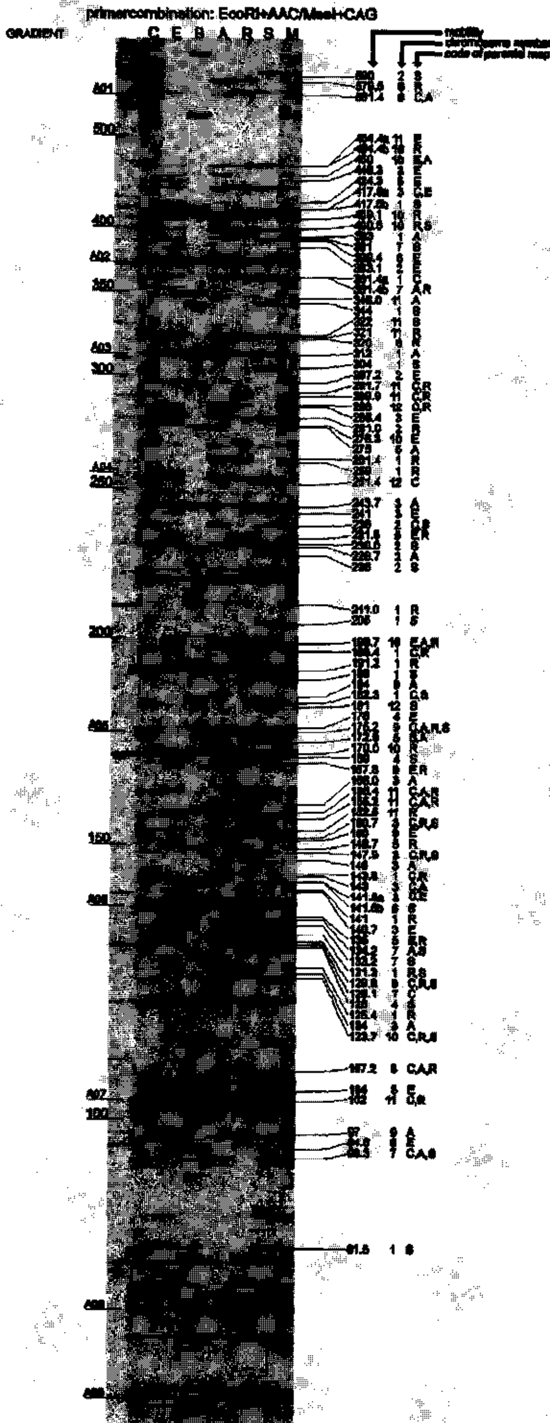

Figure 1: Presentation of the AFLP fingerprints generated with primer combination E+AAC/M+CAG. Amplification products are loaded on a 4.5% polyacrylamide gel containing an ionic gradient using 1 × TBE buffer (Vos et al. 1995) at the cathodal side and 0.5 M NaAc in 1 × TBE at the anodal side of the gel. The left lane contains a SequaMark 10 base ladder (Research Genetics, Huntsville AL USA). Subsequent lanes contain amplification products from potato clones indicated by letters explained in the legend of Table 1. The right lane contains a mixture of the amplification products generated from the six potato clones.

Table 1: Primer/enzyme combinations, numbers of markers and genotypes analysed for production of the catalogue. The restriction enzymes *Eco*RI and *Mse*I were used to generate genomic template DNA.

Primer combination	No. of common AFLP alleles	No. of AFLP markers mapped	Genotypes [a]) analysed	Number of markers for *S. bulbocastanum*
E+AAC/M+CAC	34	100	C, E, A, R, S,	15
E+AAC/M+CAG	31	94	C, E, A, R, S,	18
E+AAC/M+CCA	5	28	A, R	-
E+AAC/M+CTG	2	27	A, R	-
E+ACA/M+CAC	19	58	C, E, A, R	-
E+ACA/M+CCT	3	16	A, R	-
E+ACA/M+CGT	2	16	A, R	-
E+AAA/M+ACC	1	44	A, R	-
E+AAA/M+ACG	32	90	C, E, A, R, S,	27
E+AGA/M+CAT	16	70	C, E, A, R, B	29
E+ATG/M+CTA	43	138	C, E, A, R, S,	30
E+ATG/M+CTC	7	52	R, S	-
Total	195	733	C, E, A, R, S	119

[a])The *S. tuberosum* breeding lines harbour introgressions from the following species: clone C: *S. phureja*, clone E: *S. phureja* and *S. vernei* (Van Eck et al. 1995b), clone A: *S. tuberosum* ssp. *andigena* CPC1673, *S. vernei*, *S. venei* ssp. *balsii* and *S. oplocense*, clone S: *S. tuberosum* ssp. *andigena* CPC1673 (Rouppe van der Voort et al. 1997b). With clone B, genotype M94-106-2 of the wild species *S. bulbocastanum* is indicated (Janssen et al. 1997).

in Table 1. An example of one of the images presented in the catalogue is shown in Figure 1.

The AFLP markers are named according to the selective nucleotides at the 3' end of the primers and the electrophoretic mobility (expressed in nucleotides). Next to the marker name, information on the chromosome number is indicated as well as the parental map where the marker has been localised. The precise genomic location of a marker can be inferred from the figures showing the parental linkage maps which encompass the twelve potato chromosomes. It is noted that inherent to genetic mapping using non-inbred parents, one mapping population will result in both a maternal and a paternal genetic maps. These parental maps reflect the meiotic recombination events in either the female parent or male parent (Van Eck et al. 1995b).

Common AFLP markers

Five potato genotypes have been analysed with five AFLP primer combinations. The other seven primer combinations presented have been used to analyse only a part of the parental clones. Out of the 733 AFLP markers mapped, 195 markers were identified as common AFLP markers. These amplification products, identified in different parental

clones, were assumed to be identical alleles since 1) they were generated with identical AFLP primer combinations, 2) showed an equal electrophoretic mobility, 3) targeted the same genomic regions and 4) had identical nucleotide sequences (only a subset of the common AFLP markers was tested) (Rouppe van der Voort et al. 1997). As the vast majority of the sets of co-migrating markers appeared to be common AFLP markers, different parental maps could be aligned using these common markers as "allelic bridges" (Ritter et al. 1990) relative to the existing RFLP maps. The high incidence of common AFLP markers is explained by the highly selective and reproducible conditions under which AFLP fingerprints are generated.

The accuracy in which a genomic region can be recognised using common AFLP markers is proportional to the number of markers described in the catalogue. Obviously, the number of common AFLP markers increases with the number of primer combinations tested and the number of genotypes analysed. In Table 1, four primer combinations are presented which were applied to analyse the five *S. tuberosum* genotypes under study. With these four primer combinations 140 common AFLP markers out of a total of 422 were identified. The separate parental maps were aligned with an average of 60 common AFLP markers, an average of 5 common AFLP markers per linkage group. In our dataset, it appeared that the analysis of an additional genotype adds an average of 15 common AFLP markers to the catalogue. This is illustrated by the number of common AFLP markers identified for primer combinations E+ACA/M+CAC and E+AGA/M+CAT relative to the number of common AFLP markers found for the four most informative primer combinations (Table 1). It is estimated that by applying these four primer combinations for the construction of any new *S. tuberosum* genotype, this new map can be aligned on the basis of 60 to 75 AFLP markers. This provides a map resolution of at maximum one common AFLP marker every 13 cM.

Besides the identification common AFLP markers, which appear as polymorphic bands and segregate from at least two potato parents, a certain proportion of the common AFLP fragments will be monomorphic across a wide range of germplasm. These ever present and always monomorphic common bands are the indispensable backbone of the system to enable the orientation of AFLP images from different labs and different genotypes.

Transferability of AFLP locus information across solanaceous species

The applicability of the AFLP potato catalogue for mapping studies using other species within the genus *Solanum* was further tested by constructing a linkage map of the wild species *S. bulbocastanum*. *S. bulbocastanum* is distantly related and sexually incompatible with cultivated potato. The five most informative primer combinations (Table 1) for *S. tuberosum* were chosen to analyse an intraspecific cross of *S. bulbocastanum* (Janssen et al. 1997). Out of 408 amplification products identified for genotype M94-106-2, 119 AFLP markers segregated of which 24 markers co-migrated with markers which were mapped previously in *S. tuberosum*. The M94-106-2 parental map consisted of 13 linkage groups with a total map length of 582 cM. The lengths of

the linkage groups varied from 6 to 100 cM. However, no *S. bulbocastanum* linkage groups were identified bearing three or more common AFLP markers derived from the same chromosome.

This result indicates that application of the catalogued AFLP markers should be confined to germplasm more closely related to the species *S. tuberosum*. Comparison of the AFLP fingerprints revealed that only 24% of the AFLP fragments were similar between *S. bulbocastanum* genotype M94-106-2 and the *S. tuberosum* genotypes whereas the similarity among the *S. tuberosum* genotypes is about 58%. The diploid Central American species *S. bulbocastanum* (series *Bulbocastana*) is considered to be genetically distinct from the polyploid South American species *S. tuberosum* (series *Tuberosa*; Hawkes, 1990). The feasibility to apply the catalogue for species originating from *Solanum* series more cognate to the series *Tuberosa* *i.e.* the diploid wild species *S. sparsipilum* and *S. tarijense*, is under investigation.

Application of the AFLP catalogue

The presented catalogue can be used in at least two different ways. Firstly, by alignment of linkage groups to the chromosome map of potato, information is obtained on the genomic position of markers and genes linked to those markers. Subsequently, targeted comparative genome analysis can be performed to increase marker density in a selected genomic region (Concibido et al. 1996). By selecting a genomic interval, *e.g.* by RFLP or common AFLP markers, one can switch to the potato reference map and identify many AFLP markers which are localised in this region. These markers can either be generated by applying the respective AFLP primer combinations or by sequencing these AFLPs and converting them into cleaved amplified polymorphic sequences (CAPS; Konieczny and Ausubel 1993). CAPS markers have the advantage that they are not only suitable to switch between different potato genotypes but also across different solanaceous species (Bendahmane et al. 1997). Comparative mapping on the basis of common AFLP markers was successfully applied to localise the *Gpa2* gene, conferring resistance to the potato cyst nematode *Globodera pallida,* on chromosome *12* and to confirm the map position of the *H1* resistance gene (Rouppe van der Voort et al. 1997b).

A second application of the catalogue is the identification of the genomic region which harbour the AFLP markers which are detected via bulked segregant analysis (Michelmore et al. 1991). With this approach, AFLP markers can be identified with close linkage to the target gene without extensive segregation analyses. The map location of the target gene can be resolved when common AFLP markers are identified with one of the potato reference genotypes described in the catalogue. By use of the informative primer combinations to analyse one of the reference mapping populations, the target gene as well as anonymous markers linked to the target gene can be mapped relative to the catalogue markers. This approach was applied to map the *R2* gene conferring resistance to *Phytophthora infestans* on chromosome *4* of potato (Li et al. 1998).

The efficacy to align genetic maps via categorised AFLP markers is based on visual inspection of autoradiograms for co-migrating bands between a set of reference genotypes and any new potato genotype under investigation. In those few cases where AFLP markers mapped on different genomic positions very often subtle mobility differences were found after close re-examination of these markers on the autoradiograms. To enhance accuracy, it is therefore recommended to load amplification products generated from the reference genotypes next to fingerprints to be analysed. It also facilitates recognition of the banding patterns which can be achieved from downloaded images from the World Wide Web.

In summary, the information on AFLP markers presented in the catalogue may have various applications for genetic studies in potato. Images of AFLP fingerprints combined with detailed information on the positions of the common AFLP markers mapped are available at URL: http://www.spg.wau.nl/pv/aflp/catalog.htm. AFLP templates of the parental potato clones are available upon request, and can be used to serve as an internal reference lane in future mapping studies.

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Chapter 6

Mapping of the cyst nematode resistance locus *Gpa2* in potato using a strategy based on comigrating AFLP markers

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ABSTRACT

The nematode resistance locus *Gpa2* was mapped on chromosome *12* of potato using the information on the genomic positions of 733 known AFLP markers. The minimum number of AFLP primer combinations required to map *Gpa2* was only three. This demonstrates that a reference collection of potato AFLP markers may be a valuable tool for mapping studies in potato. By use of RFLP probes, *Gpa2* was more precisely mapped at a distal end of chromosome 12. *Gpa2* confers resistance to a distinct group of populations of the potato cyst nematode *Globodera pallida* and originates from the same potato accession as locus *H1*, conferring resistance to pathotype Ro_1 of *G. rostochiensis*. This study shows that these two nematode resistance loci are unlinked and that *Gpa2* is linked to the *Rx1* locus conferring resistance to potato virus X. The efficiency of AFLPs for genetic mapping of a highly heterozygous crop like potato is discussed and compared with the RFLP technique.

INTRODUCTION

The potato cyst nematodes *Globodera rostochiensis* (Woll.) Skarbilovich and *G. pallida* Stone are severe pests of potato. Worldwide, the average production loss is estimated to be 10 to 30% of the annual yield (Oerke et al. 1994). Crop rotation and nematicides are used to control potato cyst nematode populations but are undesirable, from an economical as well as an environmental point of view. The growth of resistant cultivars seems therefore the most effective and durable means to control potato cyst nematodes.

Classical genetic analyses of wild *Solanum* species, revealed a number of accessions with simply inherited resistance, some of which are still used to introgress nematode resistance into cultivars. Monogenic resistance was identified *e.g.* in *Solanum tuberosum* ssp. *andigena* Hawkes CPC 1673 (locus *H1*; resistance to *G. rostochiensis* pathotypes Ro_1 and Ro_4 (Huijsman 1955) and an undefined locus conferring resistance to a distinct group of populations of *G. pallida* (Arntzen et al. 1994)), *S. kurtzianum* Bitt. et Wittm. (locus *A, B*; resistance to *G. rostochiensis* pathotypes Ro_1 and Ro_2 (Huijsman 1960)), *S. multidissectum* Hawkes (locus *H2*; resistance to *G. pallida* pathotype Pa_1 (Dunnett 1961)) and *S. spegazzinii* Bitt. (locus *Fa, Fb*; resistance to *G. rostochiensis* pathotypes Ro_1 to Ro_5 (Ross 1962; Ross 1986)). More complex inheriting resistance against several populations of *G. pallida* was found, among others, in various accessions of *S. vernei* Bitt. et Wittm. (*e.g.* Goffard and Ross 1954; Ross 1986) and in *S. tuberosum* ssp. *andigena* CPC 2802 (Dale and Phillips 1982).

By use of molecular markers several of these nematode resistance loci have been placed on the genetic map of potato. Interestingly, resistance loci in potato are mapped on a limited number of genomic regions. For example, locus *GroV1,* conferring resistance to *G. rostochiensis* pathotype Ro_1, was localized in a *S. vernei* accession (Jacobs et al. 1996) in a similar region of chromosome *5* where locus *H1* (Gebhardt et al. 1993; Pineda et al. 1993) mapped. At the other arm of chromosome *5*, locus *Gpa,* conferring resistance to several populations of *G. pallida* (Kreike et al. 1994), was located and shown to be linked to RFLP markers which are in other potato genotypes linked to locus *R1* and *Rx2* (resistance to *P. infestans* and potato virus X (PVX) respectively (Leonards-Schippers et al. 1992; Ritter et al. 1991)).

So far, the majority of mapping studies in plants is still based on laborious RFLP markers. A novel DNA marker technique, called AFLP (Vos et al. 1995) has filled the need for a more efficient tool to construct dense linkage maps (*e.g.* Becker et al. 1995; Van Eck *et al.* 1995). By use of the latter method, we developed a simple procedure to place any gene of interest on the genetic map of *S. tuberosum*. In this procedure the chromosomal localization of a gene is assessed by use of comigrating AFLP markers (Rouppe van der Voort et al. 1997).

In this paper the feasibility of this strategy is demonstrated by mapping monogenic resistance against *G. pallida* identified in *S. tuberosum* ssp. *andigena* CPC 1673. In addition, the genomic position of locus *H1* is confirmed.

MATERIALS AND METHODS

Plant material: A mapping population of diploid potato (2n = 2x = 24) was obtained from a cross between the diploid clones SH82-93-488 × RH89-039-16. The female parental clone SH82-93-488 originates from a cross between the diploid *S. tuberosum* clone SH76-128-1857 and a dihaploid of clone Y66-13-628 which has cultivar Amaryl, derived from *S. tuberosum* ssp. *andigena* CPC1673, in its pedigree. Clone SH82-93-488 is the resistance donor in our population and is referred to as SH. The susceptible male parental clone RH89-039-16, will be referred to as RH.

The mapping population F_1SH×RH consisted of 194 F_1 genotypes. Seeds were germinated on moist filter papers. After germination seedlings were transferred to flats containing potting compost. Well-rooted seedlings were transplanted into plastic pots of 11 cm diameter containing a peaty soil. After six to eight weeks the top of each plant was taken for DNA extraction. In addition, three and six weeks later, stem cuttings of the potato genotypes were made for the two different nematode resistance assays. The initial seedling plants were maintained for production of tubers.

Nematodes: *G. pallida* population D383 was originally sampled from a heavily infested spot in a field at Anlo, the Netherlands. Initially, this population was encoded D234. The virulence characteristics as well as the molecular data of this population are very similar to *G. pallida* population D372 (Bakker et al. 1992; Folkertsma et al. 1996) which original code is D236. According to the scheme of Kort et al. (1977), the pathotype designation of these populations is Pa_2.

G. rostochiensis line Ro_1-19 was selected from a cross between one female and one male nematode as described previously (Janssen et al. 1990). Line Ro_1-19 is not able to reproduce on plants which contain the *H1* locus. Since avirulence to the *H1* locus is dominantly inherited at a single locus (Janssen et al. 1990), juveniles from line Ro_1-19 have the predicted genotype *AvrH1/AvrH1*.

Nematode populations were multiplied on the susceptible cultivar *S.tuberosum* ssp. *tuberosum* cv. "Eigenheimer", inoculated with approximately 200 cysts and placed in a growth chamber at 18°C and 16 h daylength.

Nematode resistance assays: Eggs and second stage juveniles (J_2) were obtained by crushing cysts which were soaked in tap water for one week. The egg/J_2 suspension was poured through a 100 µm sieve to remove debris and cystwalls. Before inoculation, three to four weeks old stem cuttings were transferred from a peat mixture to 900 g pots containing a mixture of silversand and a sandy loam fertilized with Osmocote (N-P-K granulates). Subsequently, plants were inoculated with nematodes to a final density of 5 egg/J_2 per gram soil. Of each plant genotype, three replicates per nematode source were inoculated. Six replicates of the parental clones as well as resistant and susceptible standards were included for resistance tests with each nematode source. Resistant standards were cv. Multa (resistant to D383), *S. vernei* hybrid 58.1642/4 (resistant to line

Ro_1-19) and *S. vernei* hybrid 62-33-3 (resistant to D383 and Ro_1-19). The susceptible standard was cv. Maritta. In addition, 30 tubers of cv. Saturna were inoculated with line Ro_1-19 to assess the frequency of juveniles able to develop into females on plants which contain the *H1* locus (Janssen et al. 1990). Plants were arranged in a randomized block design and grown in a greenhouse with 15°C and 25°C as minimum and maximum temperature, respectively.

After three months, cysts were recovered from the soil with a Fenwick can (Fenwick 1940) and the size of the root systems was judged on a scale of 0 to 3. Resistance data of a genotype were only recorded when at least two well-rooted plants of this genotype were available. The mean cyst numbers developed per genotype were standardized using a $\log_{10}(x + 1)$ transformation and then subjected to SAS Ward's minimum variance cluster analysis (SAS Institute Inc., Cary NC, USA). The data were separated into a resistant, unassigned and susceptible class. A chi-square test was applied to assess goodness of fit with appropriate genetic ratios.

DNA preparation and AFLP markers: DNA isolation was performed on frozen leaf tissue (Van der Beek et al. 1992) or tubers. The AFLP procedure was essentially as described by Vos et al. (1995). Template DNA was prepared using the restriction enzymes *Eco*RI and *Mse*I and pre-amplified using the "EcoRI-primer" E+0 and the "MseI-primer" M+0 (Vos et al. 1995). For selective amplification the following combinations of primers were used: E+AAA/M+ACG, E+AAC/M+CAC, E+AAC/M+CAG, E+ATG/M+CTA, E+ATG/M+CTC. AFLP data were recorded as described (Van Eck et al. 1995b). AFLP markers were designated by the primer combinations used followed by the approximate sizes of the amplification products expressed in nucleotides.

RFLP markers: To screen for polymorphisms restriction enzyme digests of genomic DNA were made using the enzymes *Eco*RI, *Eco*RV, *Hind*III, *Xba*I, *Hae*III and *Cla*I (Gibco BRL, Gathersburg, USA) using 5 units per µg DNA. Restriction fragments were separated on an 0.8% TAE buffered agarose gel (Sambrook et al. 1989) and transferred on a Hybond N+ membrane (Amersham, Little Chalfont, UK). DNA probes were radiolabeled with α-^{32}PdATP by the random prime hexamer method (Feinberg and Vogelstein, 1983). Blots were prehybridized, hybridized with radiolabeled inserts from DNA clones and washed according to Bernatzky and Tanksley (1986) using as hybridization buffer: 0.5 M phosphate (NaH_2PO_4/Na_2HPO_4; Ph 7.5), 7% SDS and 1mM EDTA and as washing buffer: 0.25 M phosphate and 1% SDS. X-Ray films (Konica, Tokyo, Japan) were exposed for 1-10 days at -80°C using one intensifying screen.

Tomato genomic DNA clones (TG-clones) and cDNA clones (CT-clones) were provided by Dr. S. D. Tanksley, Cornell University, Ithaca NY (USA.). Potato CDNA probe CP113 was kindly supplied by Dr. C. Gebhardt, Max-Planck-Institute für Züchtungsforschung, Cologne (Germany). The chromosomal positions of the TG, CT and CP markers were published in Tanksley et al. 1992 and Gebhardt et al. (1991).

RFLP clone GP34 (kindly provided by Dr. C. Gebhardt) was sequenced and

primers GP34F (CGTTGCTAGGTAAGCATGAAGAAG) and GP34R (GTTATCGTTGATTTCTCGTTCCG) were designed to screen for GP34 alleles by means of PCR (temperature file: 30 sec. 94°C, 30 sec. 62°C and 1 min. 72°C). One of the amplified alleles of GP34 cosegregated with *Gpa2* resistance. Marker NR14 which is closely linked to locus *Mi-3* in tomato was obtained from Dr. V. Williamson, University of California, Davis CA (USA). The marker was generated from tomato by PCR amplification using 0.5 μM of each of primer NR14A/R and NR14A/U. The sequences of the primers and amplification conditions were as described by Yaghoobi et al. (1995). Following amplification, the NR14 fragment was excised from the agarose gel, purified with a Glass Max Isolation Matrix System (Gibco BRL) as recommended by the manufacturer and hybridized to blots containing *Eco*RV digested DNA as described above.

Mapping: The AFLP primer combinations used in this study were previously applied to analyse two potato mapping populations in order to localize AFLP markers relative to chromosome-specific RFLP loci (Van Eck et al. 1995b and Rouppe van der Voort et al. 1997). The AFLP profiles of the parental clones of our reference populations were compared with clone SH and AFLP products of equal electrophoretic mobility which segregated in both populations were identified. Homology between a SH linkage group and one of the chromosomes identified in the reference populations was assumed when at least three comigrating AFLP markers were identified (Rouppe van der Voort et al. 1997). In this way, AFLP markers identified in the reference populations which comigrated with AFLP markers segregating from the resistant clone SH allowed identification of the chromosomes and regions thereon. The identities of the regions of interest on the SH linkage groups were confirmed by the use of RFLP markers.

Linkage analyses of pairwise recombination frequencies between markers and resistance loci were performed using JoinMap 1.4 (Stam 1993). JoinMap 1.4 tests linkage between all possible pairs of markers by calculating the logarithm of odds (LOD) score. At a LOD = 3 threshold level the test may be too conservative in assigning linkage when high recombination rates exist between two loci (Gerber and Radolphe 1994). A test for independence was therefore applied also to test for linkage between the two resistance loci.

For map construction, recombination frequencies were converted into map units (cM) by use of the Kosmabi function. A graphic representation of a map was made by the computer program Drawmap (Van Ooijen 1994).

RESULTS

Resistance testing: The inheritance of both the *Gpa2* and the *H1* gene was followed in the segregating population F_1SH×RH. For *G. pallida* population D383, the average number of cysts developed was 6 on clone SH and 286 on clone RH. Cysts counts on individual F_1 plants ranged from 0 to 681. Based on Ward's minimum variance cluster analysis (SAS

institute 1989) the class containing genotypes on which an average of < 23 cysts developed was considered as resistant and an average > 51 cysts per genotype was taken as susceptible. Genotypes containing means of 23 to 51 cysts were assigned into an intermediate class and were not used for linkage analysis.

To assess the frequency of avirulent juveniles able to develop into females on *H1* resistant plants (*i.e.* "escapers"; Janssen et al. 1990) the parental clone SH and the cultivar Saturna, both containing the *H1* locus, were tested with *G. rostochiensis* line Ro_1-19 (*AvrH1/AvrH1*). From the mean number of cysts of 0.3 (sd = 0.58) on clone SH and 2.6 (sd = 3.5) on cultivar Saturna per 5000 inoculated eggs/J_2 juveniles we assumed the maximum number of cysts due to "escapers" to be three. The mean number of cysts developed on clone RH was 80. With the inoculation density of 5000 inoculated eggs/J_2 juveniles per pot we chose the criterion of ≤ 3 cysts as resistant and > 3 cysts as susceptible. Cysts counts on individual F_1 plants ranged from 0 to 447.

Segregation of resistance loci: The resistance in parent SH to *G. pallida* population D383 resulted from a cross between a diploid susceptible *S. tuberosum* clone and diploid resistant clone which has *S. tuberosum* ssp. *andigena* CPC 1673 in its pedigree. This dominantly inheriting monogenic resistance to *G. pallida* (Arntzen et al. 1994) was therefore expected to segregate in a 1:1 ratio after crossing SH with the susceptible clone RH. A resistance test was carried out on three stem cuttings per seedling. A total of 181 F_1 genotypes were tested and based on the criteria described above, 78 genotypes were scored as susceptible and 77 as resistant (Table 1). The resistance or susceptibility of 11 genotypes could not be assessed based on Ward's minimum variance cluster analysis. From 15 genotypes, only poorly rooted plants were obtained. The segregation ratio is consistent with the assumption that a single dominant gene confers resistance to *G.pallida* population D383. We propose to name this locus *Gpa2*, in analogy to locus *Gpa* from *S. spegazzinii* (Kreike et al. 1994) which segregates with resistance to several *G. pallida* populations.

Screening for *H1* resistance was performed on a second series of three stem cuttings from 120 seedlings previously tested for *Gpa2* resistance. Segregation analysis revealed a distortion from a 1:1 segregation ratio at locus *H1* (Table 1). Comparison between segregation data obtained for locus *Gpa2* and locus *H1* by means of a 2 × 2 contingency table showed that both loci segregated independently ($X^2 = 1.73$; $P > 0.05$).

Table 1: Segregation ratios of loci *Gpa2* and *H1* in population F_1SH×RH. (*n.s.* not significant).

Locus	Resistance:susceptible F1 plants	X^2 for 1:1	Significant at:
Gpa2	77:78	0.01	$P > 0.95$ (n.s.)
H1	55:75	4.17	$0.05 > P > 0.01$

Mapping by comigrating AFLP markers: By use of five AFLP primer combinations, 322 segregating amplification products were identified in population F_1SH×RH. Out of 196 AFLP markers which segregated from clone SH, locus *Gpa2* showed linkage with eight markers (LOD scores between 3.3 and 8.2). When AFLP profiles of clone SH were compared with AFLP patterns obtained from other potato genotypes (Rouppe van der Voort et al. 1997), three AFLP markers linked with *Gpa2* comigrated with AFLP markers previously mapped on chromosome *12* (Van Eck et al. 1995b). Linkage of one of these chromosome-specific AFLP markers to *Gpa2* is presented in Figure 1,

One AFLP marker was found to be linked with locus *H1* (LOD = 9.9). This AFLP marker showed linkage with two markers (with LOD scores of 9.2 and 10.6) which comigrated with chromosome *5*-specific AFLP markers (Rouppe van der Voort et al. 1997).

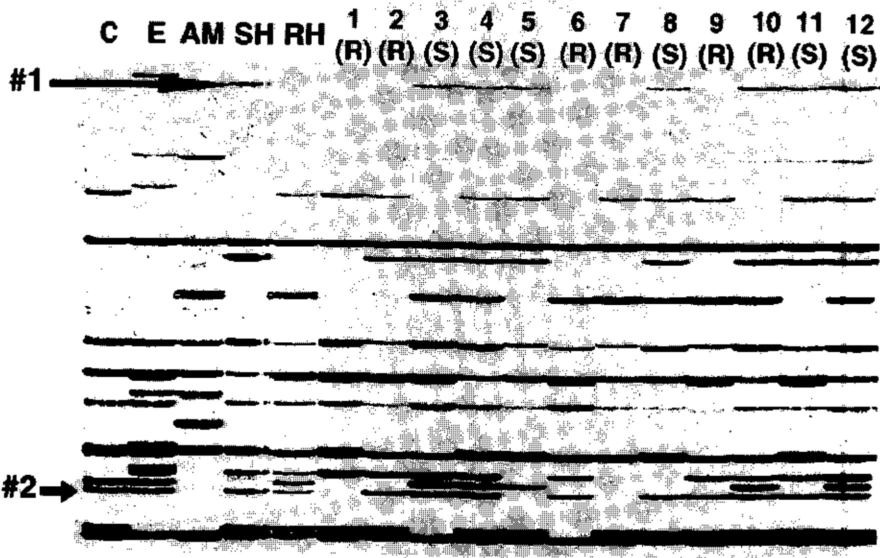

Figure 1: *Part of an autoradiogram showing AFLP markers generated from the parental genotypes SH and RH and a subset of the population F_1SH×RH. Resistance or susceptibility to G. pallida is indicated. Comigrating AFLP markers were identified by comparison with the profiles generated from genotypes C, E and AM (Rouppe van der Voort et al. 1997). #1: Marker E+AAA/M+ACG-397; linked to Gpa2 (in repulsion). The genotype marked by an asterisk shows a recombination event between marker #1 and the Gpa2 resistance. #2: Marker E+AAA/M+ACG-278; linked to Gpa2 (in repulsion) and mapped on chromosome 12 in clones C, E, SH and RH. Note that for marker E+AAA/M+ACG-278, which underlying genetic model is Aa × Aa, the heterozygous configuration is not informative. For this marker, only segregation data from lanes 1, 3, 4, 5 and 8 were used. Lane 5 represents a recombinant genotype for marker #2 and Gpa2.*

Mapping *Gpa2* and *H1*: AFLP analysis positioned locus *Gpa2* on chromosome *12*. This was confirmed by testing chromosome *12*-specific RFLP probes on population F_1 SH×RH. Labeled DNA of the RFLP clones CT99, TG360, CT100, CT79, CT129, TG68, TG180, CT19 (Tanksley et al. 1992) and the tomato PCR fragment NR14 (Yaghoobi et al. 1995) were used to probe blots containing digested DNA of the two parental clones and eight F_1 genotypes. Using the restriction enzyme *Eco*RV, RFLP markers CT100, CT129, CT79, TG68 and NR14 were shown to be linked to *Gpa2*. The other four probes, CT99, TG360, TG180 and CT19, revealed no polymorphisms between the two parental clones with the six restriction enzymes tested. Unfortunately, the segregating band identified with probe CT129 could not be scored reliably. In addition, marker GP34 was mapped by segregation analysis of an allele specific DNA fragment obtained by PCR. In this way, markers CT100, GP34, CT79, TG68 and NR14 were placed on the chromosome *12* map of clone SH.

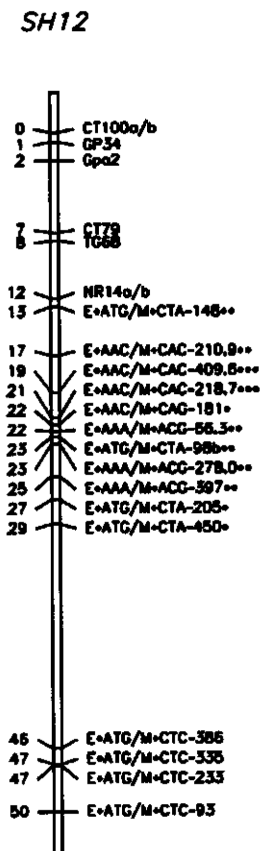

***Figure 2:** Position of the Gpa2 locus on the linkage map of chromosome 12 of clone SH. Markers which showed significant deviations on expected segregation ratios are marked by one, two or three asterisks (at 0.05 > P > 0.01; 0.01 > P > 0.001 and P < 0.001 respectively).*

The *Gpa2* locus was mapped between GP34 and CT79 (Figure 2), on a distal end of chromosome *12*. Five recombinants were identified between *Gpa2* and marker GP34 which corresponds to a map distance of 0.8 cM (LOD = 34.8). At the other side of *Gpa2*, locus CT79 mapped at a distance of 7.0 cM with a LOD score of 20.1. Marker CT100, closely linked to *Gpa2*, of which four segregating alleles could be identified is shown in Figure 3.

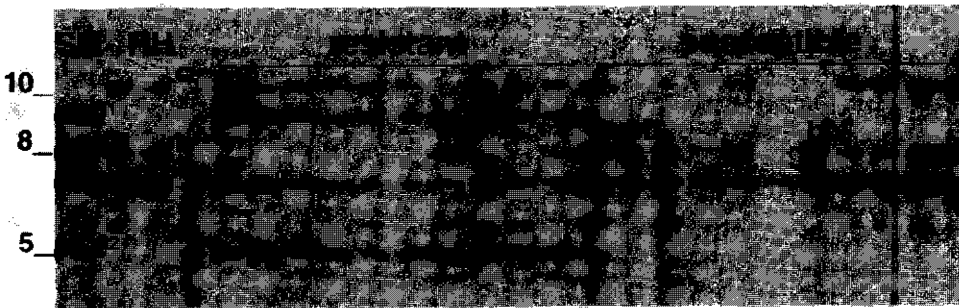

Figure 3: *Linkage of locus Gpa2 to RFLP marker CT100. Four RFLP alleles segregate in the F_1 progeny: alleles a and b derive from clone SH, alleles c and b' derive from the susceptible clone RH. Allele a is linked to the resistance. Segregation of this allele can be followed by two bands (indicated by a1 and a2). Size markers are indicated on the left in kilo basepairs.*

For both parents, significant deviations from the expected Mendelian ratios were observed for most chromosome *12* alleles. Mendelian ratios were observed only for alleles in the *Gpa2* region, segregating from clone SH. For example, at locus CT100 (Figure 3), the segregation of SH alleles *a* and *b* was as expected ($X^2 = 3.23$; $P > 0.05$). The segregation ratio of the RH alleles *c* and *b'* was strongly skewed ($X^2 = 97.2$; $P < 0.0001$) with allele *c* being the preferentially transmitted allele. Also at locus NR14, a distorted segregation ratio of the two RH alleles was observed ($X^2 = 21.7$; $P < 0.0001$), whereas the two SH alleles were equally distributed in the population ($X^2 = 0.93$; $P > 0.05$). Abnormal segregation affected all RH alleles identified on chromosome *12*. In clone SH, segregation distortion could be ascribed to a specific region, which was bracketed by markers exhibiting normal segregation ratios (Figure 2).

The map position of the *H1*-locus at chromosome *5* (Gebhardt et al. 1993) was confirmed by linkage with RFLP probe CP113 (LOD = 8.6).

DISCUSSION

The map location of *Gpa2* could be assessed readily using AFLP markers comigrating with markers which were previously placed on the genetic map of potato. It has been demonstrated that between two relatively unrelated potato clones, the majority of the AFLP fragments which have the same electrophoretic mobility are indeed identical alleles (Rouppe van der Voort et al. 1997). In retrospect, only three of the five primer combinations generated sufficient comigrating AFLP markers to map the *Gpa2* locus. This demonstrates that the 733 AFLP markers mapped so far are a valuable reference collection for other mapping studies in potato (Rouppe van der Voort et al. 1997).

The origin of the *Gpa2* resistance locus can be traced back to *S. tuberosum* ssp. *andigena* clone CPC 1673 (Arntzen et al. 1994). Out of 1200 wild *Solanum* clones, this genotype was one of the five potato clones harbouring potato cyst nematode resistance, collected in the Andean region in the early fifties (Ellenby 1952). Although *Gpa2* is unlinked to *H1* and has never been used as a selective trait in breeding programmes, *Gpa2* has been introgressed apart from *H1* into a number of commercial cultivars. The chromosome *12* region between RFLP markers CT100 and CT79 harbours also locus *Rx1* which confers extreme resistance to PVX (Bendahmane et al. 1997). *Rx1* also originates most likely from *S. tuberosum* ssp. *andigena* CPC 1673 (Ritter et al. 1991; Bendahmane et al. 1997). It is therefore hypothesized that *Gpa2* has been introgressed from *S. tuberosum* ssp. *andigena* CPC 1673 by selection for resistance to PVX in breeding programs.

The *Gpa2* locus confers specific resistance to the *G. pallida* populations D383 and D372 (Arntzen et al. (1994); herein, these populations are referred to as D234 and D236, respectively). These *G. pallida* populations are at the molecular level very similar (Bakker et al. 1992; Folkertsma et al. 1996). Both populations form a distinct group, clearly differentiated from other *G. pallida* populations found in the Netherlands. It appears that potato cyst nematode populations which extensively diverge from other populations on the basis of their molecular characteristics, can also be differentiated on the basis of their virulence characteristics (Bakker et al. 1993). This congruence between virulence and molecular characteristics holds for populations which are pathotyped Ro_1 of the species *G. rostochiensis* and Pa_1 of *G. pallida*. Ro_1 populations are differentiated from Ro_2/Ro_3/Ro_5 populations on the basis of low multiplication rates on plants containing the *H*1 locus whereas Pa_1 populations are discriminated from Pa_2/Pa_3 by multiplication rates on plants harbouring the *H*2 gene. From co-evolution theory, it has been argued that in these cases the plant-nematode interaction may follow a gene-for-gene relationship (Parrot 1981; Bakker et al. 1993; Thompson 1994). Currently, a Mendelian proof for a gene-for-gene relationship has only been provided for the interaction between *G. rostochiensis* line Ro_1-19 and the *H1* gene (Janssen et al. 1991). In line of these examples, it may be possible that a gene-for-gene relationship exists between a dominant avirulence gene present in populations D383 and D372 and the *Gpa2* gene. This hypothesis is under investigation in our laboratory by performing controlled single matings with juveniles derived from population D383.

With population D383, a maximum of 36 cysts and a mean number of 23 cysts were found on resistant genotypes. We propose two explanations for the occurrence of cysts on resistant plants. Populations D383 and D372 are probably not fixed for avirulence alleles. Hence, some virulent genotypes could be present in these populations, and these virulent nematodes are able to develop into females on *Gpa2* resistant plants. On the other hand, successful colonization of a pathogen is likely to be caused by a delayed plant defence expression rather than by complete absence of a defence mechanism (Dixon et al. 1994). Delayed plant response may result in incomplete resistance. Also with the homozygous avirulent line Ro_1-19, up to three cysts were recovered from *H1*-resistant plants. The small number of newly developed Ro_1 cysts is neither explained by *H1* gene dosage (Brodie and Plaisted 1992), nor by an exceptional high mutation frequency at the *AvrH1* locus (unpublished results). It is therefore postulated that the exceptional cases in which a syncytium matures on a resistant plant may result from differences in the timing of the resistance reaction. As the speed of a resistance response determines largely the outcome of a plant-pathogen interaction (Goodman and Novacky 1994), the time span in which the plant defence mechanism is activated may vary between the different initiated feeding cells within a plant. Consequently, in a small fraction of cells, elicitation occurs relatively late resulting in a sufficiently matured syncytium to enable female development.

As shown in this paper, RFLP markers are for potato still more accurate to identify a specific chromosomal interval. For potato and tomato, more than 1000 RFLP markers are available. Although we have mapped 733 AFLP markers on the genetic map of potato, the number of informative markers for targeting a specific genomic interval is less. Unlike RFLP markers, AFLPs are mapped as alleles rather than loci. This has important consequences for the alignment of genetic maps of different genotypes, especially for plant species like *S. tuberosum* having high levels of intraspecific variation. The ability to identify the map location of a gene with AFLPs depends on the number of comigrating AFLP markers (shared alleles) with reference genotypes. Currently, a total number of 195 comigrating AFLP markers is mapped within *S. tuberosum* by analysing five genotypes. To increase the map resolution by use of a collection of comigrating AFLP markers, additional potato genotypes and primer combinations have to analysed. Such reference collection makes the AFLP marker technique highly efficient for mapping studies within cultivated potato.

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Chapter 7

A QTL for broad spectrum resistance to cyst nematode species (*Globodera* spp.) maps to a resistance gene cluster in potato

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ABSTRACT

Broad spectrum resistance in potato to the potato cyst nematode (PCN) species *G. rostochiensis* and *G. pallida* is commonly regarded as a polygenically inherited trait. Yet, by use of QTL analysis and a selected set of PCN populations, resistance to both PCN species could be ascribed to the action of locus *Grp1*. *Grp1* confers major resistance to *Globodera rostochiensis* line Ro5-22 and *G. pallida* population Pa2-D383 and partial resistance to *G. pallida* population Pa3-Rookmaker. By use of previously characterized AFLP markers [Rouppe van der Voort et al. 1997a] *Grp1* was mapped on chromosome *5*. Cleaved amplified polymorphic sequence (CAPS) markers available for RFLP loci GP21 and GP179 revealed that *Grp1* maps on a genomic region harboring other resistance factors to viral, fungal and nematodal pathogens. The present data indicate that *Grp1* is a compound locus which contains multiple genes involved in PCN resistance.

INTRODUCTION

Characterization of complex genetic factors has been greatly facilitated by recent advances in DNA marker technology. PCR-based markers like AFLP™ (Vos et al. 1995) are efficient tools to generate dense linkage maps. With the use of these linkage maps, genomic regions containing quantitative trait loci (QTL) can be identified and accurately analyzed with the aid of statistical procedures which are amenable through publicly available computer packages (Lander and Botstein 1989; Lincoln et al. 1992; Van Ooijen and Maliepaard 1996ab). The ability to trace Mendelian loci of quantitatively inherited characters by association with DNA markers has been used to study complex agronomic traits like fruit mass, contents of soluble solids and fruit pH in tomato (Paterson et al. 1988), and polygenic disease resistances to *e.g.* bacterial wilt in tomato (Danesh et al. 1994), potato late blight (Leonards-Schippers et al. 1994) and rice blast (Wang et al. 1994).

The present study focuses on resistance to potato cyst nematodes (PCN: *Globodera rostochiensis* and *G. pallida*) which are severe pests in potato (*Solanum tuberosum* ssp. *tuberosum*). Several sources of monogenic resistance are available to potato breeders, either to *G. rostochiensis* (among others, genes *H1*; (Huijsman 1955), *Gro1*; (Barone et al. 1990) and *GroV1*; (Jacobs et al. 1996)) or to *G. pallida* (*Gpa*; (Kreike et al. 1994) and *Gpa2*; (Rouppe van der Voort et al. 1997b)). Interestingly, these resistance genes are clustered with loci involved in resistance to other plant pathogens (summarized in Leister et al. 1996). Quantitatively inheriting resistance to *G. pallida* has also been found (e.g. Goffard and Ross 1954; Ross 1986; Dale and Phillips 1982; Mugniéry et al. 1989). However, the aforementioned sources of resistance are only effective against a limited number of PCN populations.

To obtain broad spectrum resistance, breeders have accumulated an unknown number of genes in breeding lines, which are derived from a range of wild *Solanum* species (Dellaert and Vinke 1987). Although these breeding lines confer complete resistance against a large number of PCN populations, the identification of resistant offspring clones is obscured by a continuous distribution in resistance levels; no distinct classes of resistant and susceptible clones are observed. This phenomenon is typical for various sources of PCN resistance. In addition, assessment of the level of quantitatively inherited resistance against PCN is complicated by the variation in multiplication rates of the nematodes in different tests. It has been shown that variation between tests is largely due to genotype-environment interactions (Mugniéry et al. 1989). Environmental factors, such as temperature and moisture, affect the infection capabilities and the sex-ratio of the nematode (Mugniéry et al. 1984). Another difficulty in breeding for resistance against PCN is the choice of a proper test population. As with many other soil-borne pathogens, PCN have mosaic distribution patterns and even in a small area populations may have distinct genetic structures (Folkertsma 1997b). The current international pathotype scheme (Kort et al. 1977) reflects only a limited part of this variation (Bakker et al. 1993).

To obtain insight in the genetic diversity of potato cyst nematodes, more than 300 populations sampled all over Europe have been analyzed with molecular techniques (Folkertsma 1997b). At present, a diverse set of representants of the PCN metapopulation is available to evaluate the resistance spectra of potato clones.

In this paper, we report on the genetic analysis of broad spectrum resistance against PCN. By screening with diverse representants of the PCN metapopulation, the tetraploid breeding line AM78-3778 conferring broad spectrum resistance was selected and subjected to a detailed genetic study. QTL analysis revealed one major locus which is part of a resistance gene cluster on chromosome *5*. Contrary to the spectra of previously identified PCN resistance genes, this locus confers resistance to both PCN species.

MATERIALS AND METHODS

Plant material: The broadness of PCN resistance was tested in potato clones AM78-3778, 3778-14 and 3778-16. The tetraploid clone AM78-3778 contains resistance to both PCN species and is an interspecific hybrid between *S. tuberosum* and several wild potato species including *S. vernei* 24/20, *S. vernei* ssp. *ballsii* 2/1, *S. vernei* LGU 8, *S. oplocense* EBS 1786 and *S. tuberosum* ssp. *andigena* CPC 1673. Clones 3778-14 and 3778-16 are dihaploids ($2n = 2x = 24$) produced by prickle pollination of clone AM78-3778 with haploid inducer *S. phureja* clones (Hutten et al. 1994).

A mapping population of diploid potato was obtained from a cross between the clones 3778-16 × RH89-039-16. Clone 3778-16 is the resistant parent in our population and is referred to as AM. The susceptible male parent, clone RH89-039-16, will be referred to as RH. The mapping population F_1AM×RH consisted of 122 vigorous F_1 genotypes. Leaf material for DNA isolation was collected in the greenhouse at the seedling stage. Tubers for nematode tests were produced by first year clones on the field.

Nematodes: The nematode populations used in this study are listed in Table 1. The code of the population is preceded by its pathotype designation. *G. rostochiensis* lines Ro1-19 and Ro5-22 are heterogeneous populations except that they are fixed for the alleles at the avirulence and virulence locus to the *H1* resistance gene, respectively. Lines Ro1-19 and Ro5-22 were selected from controlled single nematode matings as described (Janssen et al. 1990). Pathotype Ro1 belongs to the most avirulent and Ro5 to the most virulent category of *G. rostochiensis* populations (Kort et al. 1977). The *G. pallida* populations were originally sampled from heavily infested spots in a field. Population Pa2-D383 is a relatively avirulent *G. pallida* population. Population 'Rookmaker', termed 'Pa3-Rook', is one of the most virulent *G. pallida* populations found in the Netherlands and is currently used as test-inoculum for *G. pallida* resistance in several commercial breeding programs. The virulence characteristics as well as the molecular data of these *G. pallida* populations are extensively described in Bakker et al. (1992). Nematode populations were

multiplied on the susceptible cv. Eigenheimer, inoculated with approximately 200 cysts and placed in a growth chamber at 18°C and 16-h daylength. PCN populations were stored at -80°C until use (Folkertsma et al. 1997a).

Resistance testing and data collection: Preparation of the PCN inoculum was as described by Rouppe van der Voort et al. (1997b). The resistance spectrum assay was carried out in a closed container test (Phillips et al. 1980) using 125 cc plastic containers filled with silver sand. Per container, one tuber was added and inoculated with nematodes to a final density of 5 eggs/J2 per gram soil. The containers were maintained in the dark at approximately 20°C for at least three months.

The inheritance of the resistance to populations Ro5-22, Pa2-D383 and Pa3-Rook was analyzed in mapping population F1AM×RH in three replications. Resistant standards were cv. Multa (resistant to Pa2-D383), *S. vernei* hybrid cv. Santé (resistant to Pa2-D383 and Ro5-22) and AM78-3778 (resistant to Pa2-D383, Pa3-Rook and Ro5-22). As susceptible standards cv. Eigenheimer and cv. Maritta were used. The tubers were inoculated with nematodes (final density of 5 eggs/J2 per gram soil) in 900-g pots containing a mixture of silversand and a sandy loam fertilized with Osmocote (N-P-K granulates). Plants were arranged in a randomized block design and grown in a greenhouse with 15°C and 25°C as minimum and maximum temperature, respectively.

After three months, cysts were recovered from the soil by elutriation and counted. In addition, the size of the rootsystems was classified on a scale of 0 to 3. Resistance data of a genotype were only recorded when at least three well-rooted plants of this genotype were available.

DNA marker analysis and linkage map construction: DNA isolation, AFLP analysis and data recording were done as described previously (Vos et al. 1995; Van Eck et al. 1995b). From the segregation of 408 AFLP markers, generated by use of eleven primer combinations, separate genetic maps of the parental clones were constructed (Rouppe van der Voort et al. 1997). The separate maternal AM and paternal RH maps consisted of 242 and 220 AFLP markers, respectively. These maps were aligned with the genetic map of potato by means of common AFLP markers which have been mapped relative to RFLP markers in a reference population (Van Eck et al. 1995b). Common AFLP markers were visually recognized on autoradiogram images as co-migrating bands in fingerprints generated from different genotypes using the primer combinations E+AAA/M+ACG, E+AAC/M+CAC, E+AAC/M+CAG, E+ACA/M+CGT, E+AGA/M+CAT and E+ATG/M+CTA. Genetics maps are available from URL: http://www.spg.wau.nl/pv/aflp/catalog.htm.

The PCR primer sequences and the temperature cycle files of the CAPS markers (cleaved amplified polymorphic sequences; Konieczny and Ausubel 1993) for loci GP21 and GP179 were obtained from Meksem et al. (1995). Segregating AM alleles were detected after digestion of the amplification products using the restriction endonucleases

*Dra*I for marker GP21 and *Rsa*I for marker GP179. The conditions to resolve loci GP186 and TG432 as CAPS markers were obtained from De Jong et al. (1997). The restriction endonucleases *Nci*I and *Rsa*I were used to identify segregating alleles for GP186 and TG432 respectively. The marker orders were calculated using the software package JoinMap 1.4 (Stam 1993). The primer sequences for locus CP113; 'CP113-5'1', 'CP113-3'1' and 'CP113-3'3*' were obtained from Niewöhner et al. (1995). The following temperature cycle files were applied for these markers: CP113-5'1/CP113-3'1: 3 min. 93°C followed by 30 sec. 93°C, 45 sec. 60°C, 90 sec. 72°C for 35 cycles and finished by 10 min. elongation at 72°C. CP113-5'1/CP113-3'3*: 3 min. 93°C followed by 30 sec. 93°C, 45 sec. 50°C and 90 sec. 72°C for 5 cycles after which the annealing temperature was decreased to 48°C. Also this file was completed by elongation step at 72°C for 10 min.

Statistical analysis: Analysis of variance components was carried out on $^{10}\log(x+1)$ transformed average cyst counts per plant genotype according to the following model:

$$\sigma^2_{tot} = \sigma^2_{plant} + \sigma^2_{rep}$$

where σ^2_{tot} is the phenotypic variance, σ^2_{plant} is the genetic variance among the plant genotypes, and σ^2_{rep} is the environmental variance among the replications.

The broad sense heritability was calculated according to the formulas:

$$\sigma^2_{gen} = \left(MS_{tot} - MS_{rep}\right)/3$$

$$h^2 = \sigma^2_{gen} / \left(\sigma^2_{gen} + \sigma^2_{rep}\right)$$

where σ^2_{gen} is the genetic variance among the genotypes.

The data on marker segregation of both parents were included for QTL analysis using the program MapQTL 3.0 (Van Ooijen and Maliepaard 1996ab). The markers were transferred into a biallelic code according to the manual. Three different mapping methods were applied for QTL detection. The first method was a non-parametric rank-sum test of Kruskal-Wallis (see *e.g.* Sokal and Rohlf 1995) in which the non-transformed, average cyst counts were analyzed. A threshold value of $P < 0.0001$ was used for the individual marker tests. In the second option of MapQTL, interval mapping for cross-pollinating species (CP) was applied. The likelihood that a QTL is present between two flanking marker loci is indicated by the LOD score (Lander and Botstein 1989). A LOD value of 3.0 was chosen as threshold value (Lander and Botstein, 1989). As a third method, MQM mapping (Jansen 1993; Jansen et al. 1995) was applied. The QTL with the largest effect on the trait examined was used as covariate to enhance the power in the detection of putative other QTLs. The magnitude of the marker-associated phenotypic effect is presented by the coefficient of determination (R^2) which describes the percentage of the total variance explained for by the marker genotypes in the interval mapping procedure.

RESULTS

Broadness of PCN resistance from AM78-3778: The broadness of PCN resistance present in clone AM78-3778 was assessed relative to the resistance present in the dihaploids 3778-14 and 3778-16 (AM). AM78-3778 as well as clone AM appeared to be resistant to all PCN populations tested (Table 1). Loss of resistance to the Pa3-populations was observed in clone 3778-14. The diploid clone RH89-039-16 (RH) was susceptible to all populations tested.

Table 1: Broadness of resistance in AM78-3778 derived clones. Presented are average numbers of cysts recovered in a closed container test.

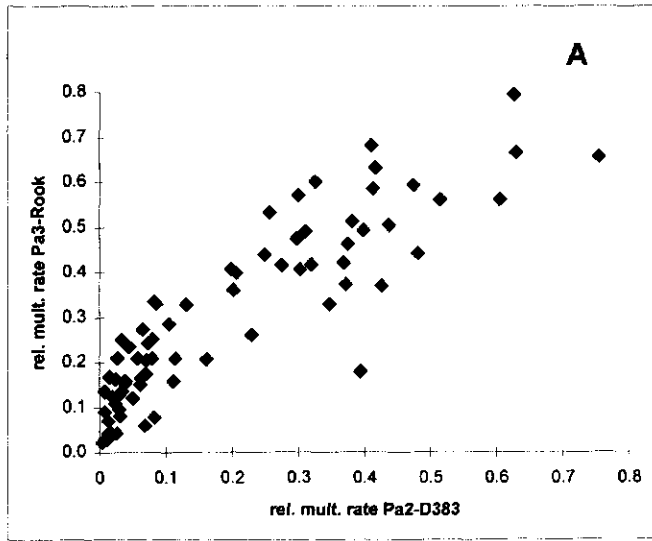
Potato clone	Ro1-19	Ro5-22	Pa2-D383	Pa2-D350	Pa2-HPL1	Pa3-1097	Pa3-Rook	Pa3-74.768.20
AM78-3778	2	10	0	0	2	2	2	4
3778-16	2	7	0	0	1	4	4	13
3778-14	n.d.	n.d.	1	9	7	135	108	73
RH89-039-16	189	98	101	132	146	240	153	166

n.d.: not determined

Inheritance of the resistance: The average numbers of cysts developed on the parental genotypes are shown in Table 2. Analysis of variance on normalized cyst counts revealed that the genetic variance for both *G. pallida* and *G. rostochiensis* resistance was significant ($P < 0.0001$). No significant differences in cyst numbers were found among the blocks of replicates. The broad sense heritabilities, listed in Table 2, indicate that the variation in cyst counts was hardly affected by environmental factors and root system development. It is noted that the values for skewness and kurtosis sometimes deviate from the test criteria on normally distributed ^{10}log(x+1) transformed cyst numbers (Snedecor and Cochran 1967). The analyses on these data may therefore be slightly biased.

Table 2: Results of the quantitative analysis of PCN resistance as measured by counted cyst numbers of the respective PCN populations. Skewness and kurtosis of ^{10}log(x+1) transformed cyst numbers, heritability of the specific resistance, the map location of the QTLs with their nearest marker, the *P* value of the nearest marker in a Kruskal-Wallis test, the LOD score and the R^2 at the QTL position are given. Abbreviations: # cysts: average number of cysts, h^2 : heritability, R^2 : percentage of the total variance explained by the marker genotypes, Marker : marker nearest to QTL.

Trait	# cysts AM	# cysts RH	skewness	kurtosis	h^2	Marker	R^2	*P* value	LOD
Pa2-D383	5	662	- 0.45	- 0.87	0.86	GP179	66%	< 0.0001	16.8
Pa3-Rook	30	1067	- 0.70	0.19	0.83	GP179	45%	< 0.0001	11.7
Ro5-22	5	3961	- 0.41	- 1.32	0.83	GP21	77%	< 0.0001	17.9
Rootsystem	-	-	-	-	0.086	-	-	-	-



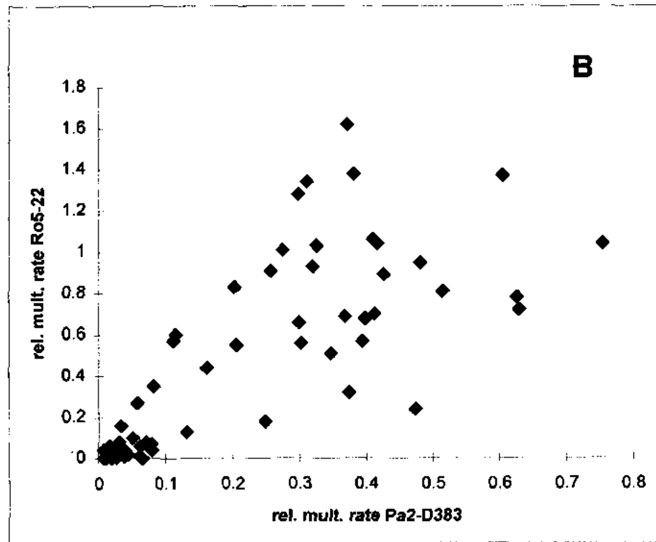


Figure 1: *Comparison of relative multiplication rates (rel. mult. rates) of different PCN inocula on F1-genotypes. The relative multiplication rates on F1-genotypes are expressed by the number of newly developed cysts devided by the number of cysts developed on the susceptible parent RH. In Figure 1a the comparison of rel. mult. rates on F1-genotypes is shown between populations Pa2-D383 and Pa3-Rook; in Figure 1b between populations Pa2-D383 and Ro5-22.*

Figure 1a shows that in the progeny, the resistance to population Pa2-D383 is correlated with resistance to population Pa3-Rook. A decrease of the relative multiplication rate of population Pa2-D383 on a F1-genotype is associated with a decrease in the relative multiplication rate of population Pa3-Rook. For example, the set of F1-genotypes where relative multiplication rates between 0 and 0.08 (actual cyst numbers between 0 and 56) for population Pa2-D383 were found, showed relative multiplication rates between 0 and 0.2 (cyst numbers between 0 and 140) for population Pa3-Rook. Similarly, it is shown that the resistance to *G. pallida* Pa2-D383 is correlated with resistance to *G. rostochiensis* line Ro5-22 (Figure 1b).

AFLP markers: By scoring resistance to Pa2-D383 as a monogenic trait using the arbitrary criterion of genotypes containing < 56 cysts as being resistant and genotypes containing > 56 as being susceptible, linkage (at LOD > 3.0) was found with six AFLP markers localized on the map of genotype AM. These AFLP markers reside on chromosome *5* as determined by their linkage with previously mapped common AFLP markers. Common AFLP markers are visually recognized as co-migrating bands in fingerprints of potato genotypes which have also been analyzed with chromosome specific RFLP markers (Rouppe van der Voort et al. 1997a). Pa2-D383 resistance could not be precisely mapped on chromosome *5*, apparently due the variance to high to be explained by a monogenic analysis. A more profound analysis of the data was therefore performed by a QTL approach (see below). In addition, resistance to Pa3-Rook as well as resistance to Ro5-22 could only be mapped on chromosome *5* by means of Pa2-D383 resistance as the bridging marker. Nevertheless, monogenic analysis of the datasets indicates the presence of genetic factor(s) allocated on chromosome *5*. A detailed description of the AFLP markers, used for alignment of potato maps are given at URL: http://www.spg.wau.nl/pv/aflp/catalog.htm.

CAPS markers: CAPS markers were tested to identify additional markers at both arms of chromosome *5*. The loci GP21 and GP179 (Gebhardt et al. 1991; Meksem et al. 1995) were detected after digestion of the amplification products using the restriction endonucleases *Dra*I for marker GP21 and *Rsa*I for marker GP179 (Figure 2). The marker orders on chromosome *5* for loci GP21, GP179, GP186 and TG432 are in agreement with the *S. tuberosum* maps of Gebhardt et al. (1991) and De Jong et al. (1997). Unfortunately, locus CP113 could not be mapped as a CAPS marker in population F1AM×RH. Both primer combinations (Niewöhner et al, 1995) produced a monomorphic amplification product even after digestion using a series of 12 four-basepairs recognizing restriction endonucleases.

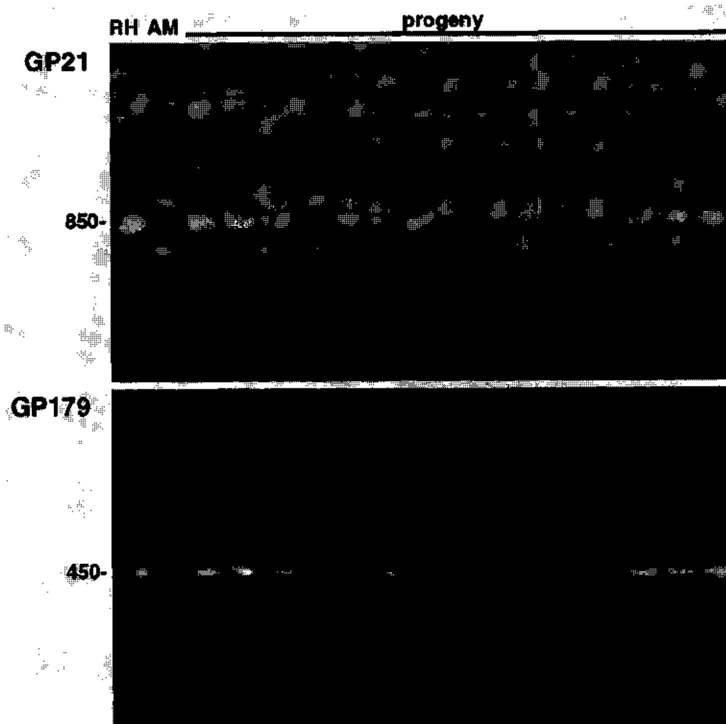


Figure 2: *CAPS analysis of RFLP markers GP21 and GP179. Linkage of both CAPS markers is shown by the profiles generated from the susceptible parent RH, the resistant parent AM and a subset of their progeny. The segregating AM alleles are linked in coupling phase with an allele conferring PCN resistance. Molecular weights of the DNA fragments are given on the left.*

QTL mapping: The computer program MapQTL (Van Ooijen and Maliepaard 1996ab) was used to analyze both the resistant and the susceptible parental dataset. The Kruskal-Wallis test revealed significant associations between resistance against the *G. pallida* and *G. rostochiensis* populations and chromosome *5* markers segregating from clone AM. The highest significance levels were found at markers GP21 and GP179 for the three inoculum treatments (Table 2). Further on this chromosome, a second gradient in the Kruskal-Wallis test statistic was observed presumably due to missing values for markers mapped on this region.

The LOD profiles of the interval mapping are presented in Figure 3. This figure shows high LOD-values for the interval flanked by markers GP21 and GP179 (3 cM), indicating a large effect on the resistance for the three PCN populations tested (see also Table 2). From the interval mapping, it was unclear whether the high LOD values for other chromosome *5* intervals were the result of their linkage with the GP21-GP179 interval or a second QTL. However, the MQM mapping method revealed no additional statistically significant effects for PCN resistance at other genomic intervals.

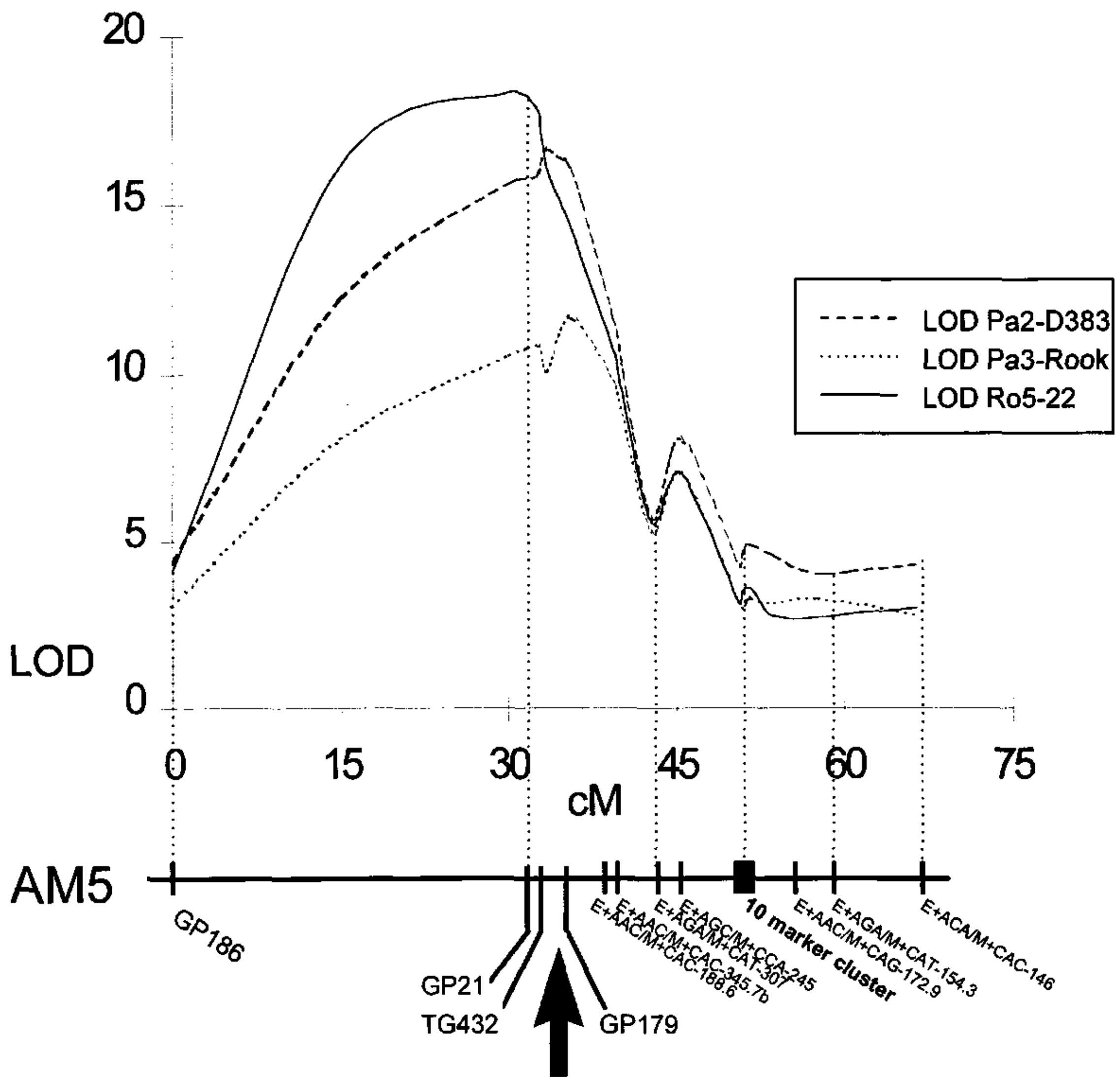


Figure 3: *LOD plot for PCN resistance on chromosome 5 of genotype AM. The position of the QTL on the map is indicated by an arrow.*

DISCUSSION

The results of the QTL mapping show that a locus with large effects on the resistance to both PCN species is localized on the genomic region possessing loci GP21 and GP179. This locus confers major resistance to *G. rostochiensis* line Ro5-22 and *G. pallida* population Pa2-D383 as well as partial resistance to *G. pallida* population Pa3-Rookmaker. On the potato map, the GP21-GP179 region is known to contain a cluster of resistance genes encoding specificities to many different plant pathogens, *e.g.* to the fungus *Phytophthora infestans,* (gene *R1* and a major QTL; (Leonards-Schippers et al. 1992, 1994)), to potato virus X (extreme resistance *Rx2*; Ritter et al. 1991, hypersensitive resistance *Nb*; De Jong et al. 1997) as well as to *G. pallida* (locus *Gpa*, (Kreike et al. 1994)). Although mechanistically considered to be a different class of resistance, a QTL involved in trichome-mediated insect resistance (Bonierbale et al. 1994) resides also in this region.

The presence of quantitative resistance as well as qualitative resistance on the same chromosome region opens the possibility that resistance genes and the genes underlying QTLs are alleles of the same genetic locus. This assumption fits with the hypothesis that qualitative phenotypes are extreme, mutated allelic variants at a quantitative trait locus (Robertson 1985). Moreover, major resistance (*R*) genes cloned from several plant species share striking structural similarities despite their intimate interaction with a diversity of pathogen species. These major genes seem to be members of large multigene families, arranged in large arrays of complex, evolutionary related but different loci having different specificities (reviewed in Baker et al. 1997).

In this context it seems likely that the QTL for PCN resistance mapped in this study is a compound locus containing different but related *R*-genes for PCN resistance. The diploid clone AM, used to map PCN resistance, is derived from AM78-3778, a tetraploid clone which combines the resistance introgressed from many separate wild *Solanum* sources (Dellaert and Vinke 1987). As neither of these wild species contain resistance to both *G. rostochiensis* and *G. pallida* the underlying genetic model for the locus identified in AM78-3778 probably includes more than one gene. Since these genes have been separately introgressed, the in coupling linkage phase of these genes should be explained by the many generations involved in breeding of clone AM78-3778 which may have resulted in fortuitous recombination events producing multiple *R*-genes on the same homologous chromosome. We propose to name the PCN resistance locus *Grp1* (for *G. rostochiensis* and *G. pallida* resistance) until future research will enable us to ascribe the resistance of *Grp1* to the action of different genes.

The resistance to Pa3-Rook seems to involve additional loci given the fact that seven offspring clones harbor resistance levels comparable to that of parent AM. In addition, *R*-genes which confer complete resistance at the level of the individual nematode may have the appearance of quantitatively inherited resistance genes when heterogeneous pathogen populations are used in the resistance test. The occurrence of the latter genetic model is not inconceivable, because resistance tests rely predominantly on screening with field

populations. These field populations are often not homogeneous for virulence traits but are mixtures of virulent and avirulent genotypes. In case a PCN population is not homogeneous for the virulence trait examined, a single *R*-gene operating on the basis of a classical gene-for-gene relationship will confer partial resistance against the population as a whole, whereas at the level of the individual the *R*-gene will confer absolute resistance against the matching avirulent genotype. Formal proof for a gene-for-gene relationship has so far only been obtained for the interaction between *G. rostochiensis* and the *H1* gene from *S. tuberosum* ssp. *andigena* CPC1673 (Janssen et al. 1991), but it is likely that various other PCN resistance genes act in a similar way. It is therefore hypothesized that for complete resistance to Pa3-Rook as observed in AM78-3778 only a few loci are involved. A precedent consistent with this hypothesis is that in the dihaploid genotype 3778-14, Pa3 resistance was lost whereas the Pa2 resistance was maintained. Thus, complete resistance can be obtained by accumulating *R*-genes with specificities which match with the different avirulent genotypes in a heterogeneous field population.

The question of whether the resistance to Pa2-D383 and Pa3-Rook is mediated by the same *Grp1* allele remains unanswered by the results of this study. No recombination was found between Pa2-D383 and Pa3-Rook resistance among the three GP21/GP179 recombinant genotypes identified in population F1AM×RH. Kreike et al. (1994) found recombination between Pa2 and Pa3 resistance as well as between markers in the same genomic interval. This indicates that *Gpa* may be a compound locus indeed. Partial overlap has been found in the resistance spectra of *Grp1* and *Gpa*; *Grp1* confers resistance to populations of both PCN species and only partial resistance to Pa3-Rook whereas *Gpa* confers resistance to all *G. pallida* populations tested so far but not to *G. rostochiensis* (Kreike et al. 1994; P. Wolters, personal communication). Therefore, *Grp1* should be considered as being different to *Gpa* but may have allele(s) in common with *Gpa*.

In this report it is shown that by testing with a diverse set of representants of the PCN metapopulation, broad spectrum PCN resistance in AM78-3778 is determined by the action of different genes at a compound locus. The *Grp1* locus harbors resistance to both PCN species. Although it confers incomplete resistance to *G. pallida*, it is expected that complete resistance can be achieved by introgression of *R*-genes with complementary specificities. Current breeding strategies for PCN resistance rely on trial and error approaches. However, the availability of a representative set of pathotype populations and the development of MAS assays will allow a more directed approach towards achievement of complete PCN resistance in commercial cultivars by combining a genes with complementary specificities.

ACKNOWLEDGEMENTS

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Chapter 8

Tight physical linkage of the nematode resistance gene *Gpa2* and the virus resistance gene *Rx* on a single introgression segment from the wild potato species *Solanum tuberosum* ssp. *andigena* CPC 1673

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Submitted for publication

ABSTRACT

The occurrence of different plant resistance (*R*-) genes on a single introgression segment which confer resistance to taxonomically unrelated pathogens is rarely observed. In this paper we describe a gene cluster in potato which harbors loci *Gpa2* and *Rx*, conferring resistance to the potato cyst nematode *Globodera pallida* and potato virus X (PVX), respectively. By screening two mapping populations with 10 CAPS (cleaved amplified polymorphic sequences) markers derived from the ends of overlapping bacterial artificial clones (BAC), it is demonstrated that *Gpa2* and *Rx* are tightly linked and located on the short arm of chromosome *12*. Among a total progeny of 3386 clones, only one genotype was identified which displays a recombination event between *Gpa2* and *Rx*. *Gpa2* and *Rx* are located at 0.02 cM distance within a stretch of 220 kb of DNA. Evidence is provided that the introgression segment harboring *Gpa2* and *Rx* originates from the wild species clone *Solanum tuberosum* ssp. *andigena* CPC 1673. The evolutionary relationship between *Gpa2* and *Rx* is discussed.

* These authors contributed equally to this chapter

INTRODUCTION

Most plant resistance genes (*R*-genes) are dominantly inherited and are often involved in resistance processes which are characterized by a hypersensitive response (HR) (Staskawicz et al. 1995; Crute and Pink 1996; Hammond-Kosack and Jones 1996). Two distinct genetic arrangements have been found among *R*-loci: either single loci of which many different alleles occur in the germplasm (Bent et al. 1994; Mindrinos et al. 1994; Lawrence et al. 1995; Grant et al. 1995) or large arrays of different genes which occur at compound loci forming *R*-gene clusters (Jones et al. 1994; Martin et al. 1993, 1994; Dixon et al. 1996; Anderson et al. 1997; Parniske et al. 1997; Song et al. 1997). The occurrence of different *R*-genes on the same genomic location in different species of a plant genus has been ascribed to the common origin of resistance gene families (Baker et al. 1997). This is exemplified by the recent characterization of the *Cf-4/9* locus of tomato. The resistance genes *Cf-4* and *Cf-9* originate from the wild species *Lycopersicon hirsutum* and *L. pimpinellifolium* respectively and confer resistance to different races of the fungal pathogen *Cladosporium fulvum*. Sequencing of the entire region in both the wild and the cultivated species revealed several (active) resistance gene homologs indicating that frequent sequence exchange between these tandemly repeated genes created novel resistance gene specificities (Thomas et al. 1997; Parniske et al. 1997). Similar evolutionary processes may underlie the generation of novel resistance gene specificities against different pathogen species. For example, a complex locus is known on chromosome *5* of potato. This cluster comprises of at least five *R* loci: *R1* which bestows resistance to *Phytophthora infestans* (Leonards-Schippers et al. 1992), *Nb* conferring a HR type of resistance and *Rx2* conferring extreme resistance to potato virus X (De Jong et al. 1997; Ritter et al. 1991) and *Gpa* and *Grp1* conferring resistance to the potato cyst nematode (Kreike et al. 1994; Rouppe van der Voort et al. 1998). Also these loci originate from different genetic backgrounds.

At present, it is unknown how *R*-loci, conferring resistance to taxonomically unrelated pathogens, are physically related. Resistance gene like sequences at compound *R*-loci have frequently been demonstrated for a number of plant species (Martin et al. 1993, 1994; Dixon et al. 1996; Leister et al. 1996; Anderson et al. 1997; Parniske et al. 1997; Song et al. 1997). However, it is impossible to predict from the DNA sequence if multiple genes are functional to different pathogen species. Only one example is known for tomato, in which resistance loci to the root knot nematode *Mi* and the aphid resistance locus *Meu1* are tightly linked and likely to be derived from the same 650 kb segment of the wild species *Lycopersicon peruvianum* (Kaloshian et al. 1995).

Here, the identification of a *R*-gene cluster in potato is described. Two *R*-genes, *Gpa2* which bestows a specific resistance response to a small set of populations of the potato cyst nematode *Globodera pallida* (Rouppe van der Voort et al. 1997) and *Rx* which confers extreme resistance to PVX (Ritter et al. 1991, Bendahmane et al. 1997), are mapped on the short arm of chromosome *12*. Evidence is provided that both genes are physically tightly linked and that they reside on an introgression segment which has been

introduced into a number of European potato cultivars from a single wild *Solanum* accession.

MATERIALS AND METHODS

Plant material: The tetraploid (2n = 4x = 48) progeny of selfed Cara (S1-Cara) was initially constructed for fine mapping of *Rx* (Bendahmane et al. 1997). This population was extended to a total of 2,788 genotypes. In addition a diploid mapping population (2n = 2x = 24) was investigated derived from a cross between the diploid clones SH83-92-488 and RH89-039-16. This population, encoded F1SH×RH, has been used for mapping the *Gpa2* gene (Rouppe van der Voort et al. 1997) and consisted of a total of 598 genotypes. The resistant parent SH83-92-488 and the susceptible parent RH89-039-16 will be referred to as SH83 and RH89 respectively.

Seed from a selfed offspring of *Solanum tuberosum* ssp. *andigena* CPC 1673 encoded CPC 3520 was obtained from the Commonwealth Potato Collection, Scottish Crop Research Institute, Dundee, UK. The cultivars 'Multa', 'Alcmaria', 'Amaryl', 'Marijke' and 'Saturna' having *S. tuberosum* ssp. *andigena* CPC 1673 in their pedigree, were derived from the collection of the Centre for Plant Breeding and Reproduction Research (CPRO-DLO) Wageningen, The Netherlands. It is noted that the background of parent SH83 contains cultivar 'Amaryl'.

Resistance tests: The PVX resistance test was carried out using a cDNA of the PVX_{CP4} isolate (Goulden et al. 1993). Potato plants were graft-inoculated with scions of *Lycopersicon esculentum* cvs. Ailsa Craig or Money Maker systemically infected with PVX_{CP4}. Northern blots were prepared from total RNA isolated from newly formed potato shoots 3-4 weeks post-inoculation (Bendahmane et al. 1997). Extreme PVX resistance or susceptibility was determined by the presence or absence of a hybridization signal on Northern blots probed with ^{32}P-labelled cDNA of PVX_{CP4} (Chapman et al. 1992). Three replicates per genotype were assayed.

For the *Gpa2* test *G. pallida* population Pa_2-D383 was used (Rouppe van der Voort et al. 1997). The nematode resistance assays were performed on plants derived from *in vitro* stocks, stem cuttings or tubers. *In vitro* plants were transferred from MS medium containing 3% saccharose to a mixture of silversand and sandy loam under a moist chamber for one week. Two to four weeks after planting, plants showing vigorous growth were inoculated with nematodes. Assays were further performed as described for stem cuttings and tubers (Rouppe van der Voort et al. 1997). Population Pa_3-Rook with different virulence characteristics as Pa_2-D383 (Bakker et al. 1992) was used to confirm the specificity of *Gpa2* resistance in tested plants.

DNA manipulations: Genomic DNA was isolated from frozen leaves (Van der Beek et al. 1992) or from seedlings (Bendahmane et al. 1997). CAPS (cleaved amplified

polymorphic sequences; Konieczny and Ausubel 1993) markers were derived from a sequenced insert of RFLP clone GP34 or from cloned AFLP markers (coded by a 'IPM' prefix; Bendahmane et al. 1997). A third type of CAPS marker was generated from the flanking sequences of BAC clones (coded by a number followed by the letter 'R' or 'L', for left or right terminus respectively). The construction of a BAC contig and production of the CAPS marker sequences and the primer sequences of the unpublished CAPS markers will be described elsewhere (A.B. K.K and D.B. submitted for publication).

Analysis of recombination: Estimates of recombination frequencies and their standard errors were calculated with the aid of the program Linkage-1 (Suiter et al. 1983) by choosing the appropriate genetic model for each cross. Data for the non-recombinant class of genotypes were set for either a 3:1 segregation ratio for population S1-Cara or a 1:1 segregation ratio for population F1SH×RH since only strongly skewed segregation ratios will influence estimates of recombination frequencies notably (Säll and Nilsson, 1994; Manly, 1994). A chi-square test was used to test for differences in recombination frequencies between the marker intervals. The chi-square test criterion was determined from the recombinant and non-recombinant classes for each marker interval. Differences (rejection of the null hypothesis) were significant when the test criterion was greater than the $X^2_{[.05]}$ value.

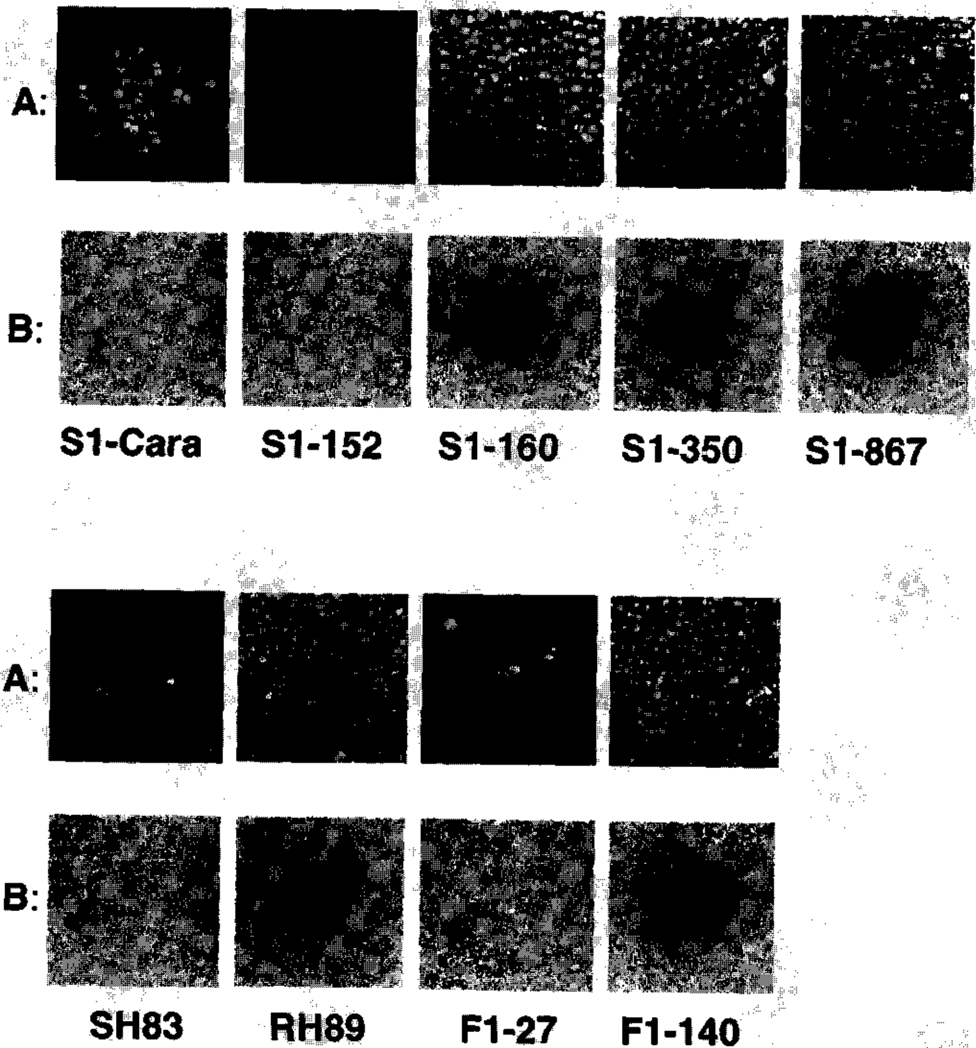

Figure 1: *Cosegregation of Gpa2 and Rx resistance demonstrated on the parental clones Cara, SH83 and RH89 as well as on four offspring genotypes of population S1-Cara (S1-152, S1-421, S1-160, S1-1189) and two genotypes of population F1SH×RH (F1-27, F1-140). A: cysts recovered from the roots of one replicate of each plant. Average numbers of cysts and their standard errors for the resistant and susceptible class are 37 ± 7 and 715 ± 28 respectively. Magnification 5×. B: hybridization signal on Northern blots of total RNA of the same set of plants infected with* PVX_{CP4}.

RESULTS

Cosegregation of *Gpa2* and *Rx* resistance: To confirm the assumed linkage between *Gpa2* and *Rx* (Rouppe van der Voort et al. 1997), a pilot experiment was carried out in which the segregation of both genes was followed in the tetraploid population S1-Cara and the diploid population F1SH×RH. Potato genotypes Cara and SH have the wild accession *Solanum tuberosum* ssp. *andigena* CPC 1673 in common and both genes were localized on the same genomic region of potato chromosome *12* (Bendahmane et al. 1997; Rouppe van der Voort et al. 1997). The tests for *Gpa2* and *Rx* resistance were performed on the parental genotypes Cara, SH83 and RH89, four S1 genotypes which were recombined in a 1.21 cM interval between markers GP34 and IPM5 (Bendahmane et al. 1997) and two F1 genotypes which harbored cross-over events in a 6 cM interval between markers GP34 and CT79 (Rouppe van der Voort et al. 1997).

The resistance tests showed a clear reduction in the number of cysts of *G. pallida* population Pa_2-D383 on genotypes which were resistant to PVX_{CP4} (Figure 1). The number of cysts developed on the resistant S1-Cara genotypes appeared to be slightly higher than the number of cysts found on the resistant genotypes of population F1SH×RH. However, a considerable reduction in size of these cysts was observed as compared to the cysts developed on a susceptible genotype. This observation was corroborated after comparing the number of eggs per cyst developed on resistant and susceptible genotypes. Average cyst contents were determined from at least 30 cysts (if possible) and subjected to a *t*-test. A significant difference (at $P < 0.05$) was found between the average number of eggs per cyst developed on Cara, SH83 and cv. Multa (resistant control), and average egg contents per cysts recovered from genotype S1-350, RH89 and cv. Eigenheimer (susceptible control). Resistance tests using *G. pallida* population Pa_3-Rook (results not shown) show that cv. Cara is susceptible to this nematode population, indicating a specificity for the *G. pallida* resistance in population S1-Cara.

Although limited numbers of S1-Cara and F1SH×RH genotypes were tested for resistance to *G. pallida* population Pa_2-D383 and PVX respectively, the crossover events in the tested plants (see Figure 2) allow one to conclude that *Gpa2* and *Rx* cosegregate in both mapping populations (with a maximum probability of $P = 1/64$ that the observed linkage could be explained by chance). The S1-Cara recombinants were previously used to delimit the *Rx* interval between markers IPM3 and IPM5 (Bendahmane et al. 1997). Cosegregation of *Gpa2* with *Rx* indicates therefore that *Gpa2* also resides in this region.

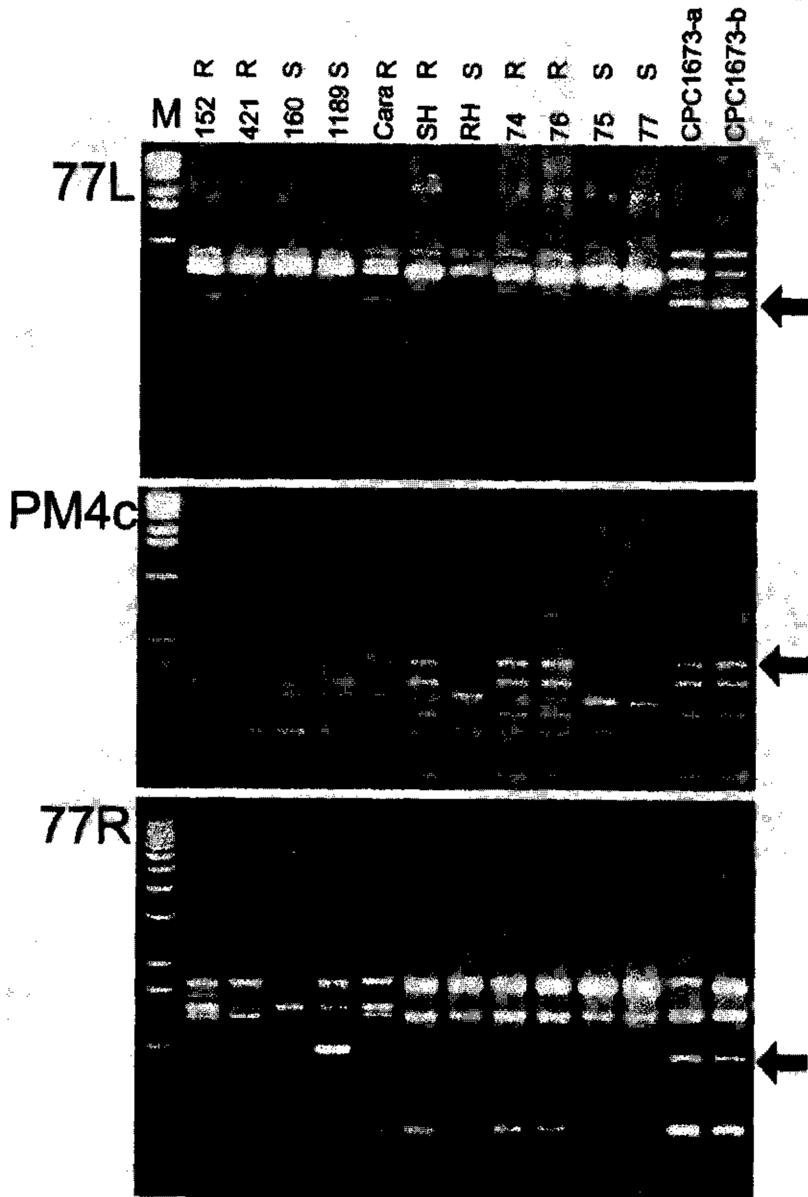

Figure 2: *CAPS marker analysis on a set of S1-Cara and F1SH×RH progeny as well as a series of S. tuberosum ssp. andigena CPC 1673 derived potato clones. A: Marker 77L bracketing the left border of the Rx interval by clone SH83. B: Marker IPM4c bracketing the right border of the Rx interval and the left border of the Gpa2 interval. C: Marker 77R bracketing the left border of the Gpa2 interval. See also Figure 3.*

Origin of *Gpa2* and *Rx* resistance: To address the question of whether *Gpa2* and *Rx* were jointly introgressed from the same wild species, a subset of the progeny of S1-Cara and F1SH×RH, two potato clones derived from a selfing of the original wild accession (abbreviated as CPC1673-a and CPC1673-b) and nine potato cultivars harboring introgressions from *Solanum tuberosum* ssp. *andigena* CPC 1673 (hereafter referred to as CPC1673) were evaluated for the presence of common DNA marker alleles in the GP34-IPM5 region of chromosome *12*. CAPS markers were derived from a high resolution physical map around the *Rx* locus constructed for cultivar Cara. As the primers for these CAPS markers may amplify various sequences scattered all over the genome, only the DNA fragments *in cis* to *Rx* were scored. The CAPS marker profiles were highly similar for the selfed CPC1673 genotypes and the analyzed potato cultivars harboring introgressions from CPC1673

CAPS marker alleles *in cis* to *Rx* were only identified in regions which appeared to be of CPC1673 origin (Table 1). Among the seven CPC1673 cultivars tested, five differences in the size of an introgressed region of 0.9 cM were observed. These differences in introgression segment lengths led unambiguously to the same marker order. The introgressed region in the *Gpa2/Rx* containing genotypes Amaryl, SH83 and Marijke was interrupted between marker 77L and IPM4c. Therefore, the genomic interval containing both resistance genes could be narrowed down to markers 77L and IPM5 (Table 1).

Genetic distance between Gpa2 and Rx: For fine mapping of *Gpa2*, 2,788 S1-Cara genotypes were assayed for recombination events in the IPM3-IPM5 region. In addition, 598 genotypes from population F1SH×RH were subjected to a GP34/IPM5 marker screening as marker IPM3 was not informative in this population. The CAPS marker screening provided a total of 20 recombinants in population S1-Cara and 9 recombinants for population F1SH×RH. These recombinants were subsequently tested for all markers available in the IPM3-IPM5 region as well as for *Gpa2* resistance. This analysis showed that *Gpa2* is located between markers IPM4c and 111R. Interestingly, among the 2,788 S1-Cara genotypes and 598 F1SH×RH genotypes tested, only one genotype, S1-761, was identified in which a recombination event had occurred between *Gpa2* and *Rx*. On the other side of *Gpa2*, genotype S1-B811 could be used to identify marker 111R as a flanking marker for the *Gpa2* interval. Marker orders deduced from the analysis of F1SH×RH corresponded to those found in population S1-Cara. Therefore, estimates of recombination frequencies deduced from both populations were merged to obtain an estimate of the average recombination value for each marker interval (Table 2). Significant differences in recombination frequencies were observed between marker intervals GP34-77L, 73L-218R and the intervals in the region 77L-73L, even when the total number of recombinants and non-recombinants were combined and compared with those of marker intervals GP34-77L and 73L-218R. The graphical genotypes (Young and Tanksley, 1989) shown in Figure 3 display the boundaries of the *Gpa2* and *Rx* intervals.

Table 1: Potato clones having *S. tuberosum* spp. *andigena* CPC1673 in their pedigree (with the exception of clone RH89) tested on the presence of chromosome *12* specific CAPS alleles. Presence or absence of a CAPS marker band that cosegregates with resistance in populations S1-Cara and F1SH×RH is indicated by "+" or "-" respectively. The order of the presented CAPS markers corresponds to the marker order on chromosome *12*.

Clone	*Gpa2*	*Rx*	IPM3	191L	77L	IPM4c	77R	IPM4a	111R	73L	218R	IPM5
CPC1673-a	n.d.	n.d.	+	+	+	+	+	+	+	+	+	+
CPC1673-b	n.d.	n.d.	+	+	+	+	+	+	+	+	+	+
Cara	R[a]	R[b]	+	+	+	+	+	+	+	+	+	+
Alcmaria	R[c]	R[d]	-	+	+	+	+	+	+	+	+	+
Multa	R[a]	n.d.	-	-	+	+	+	+	+	+	+	+
SH83	R[a]	R[b]	-	-	-	+	+	+	+	+	+	+
Amaryl	R[c]	R[d]	-	-	-	+	+	+	+	+	+	+
Marijke	R[c]	R[d]	-	-	-	+	+	+	+	+	+	+
Saturna	S[a]	S[d]	-	-	-	-	-	-	-	-	-	+
RH89	S[a]	S[b]	-	-	-	-	-	-	-	-	-	-

[a] As determined by cyst counts on at least three replicates; [b] As determined by a slot blot assay on at least three replicates; [c] Data from Arntzen et al. (1994); [d] Data from Anonymous (1991), 'R' indicates extreme resistance.

Table 2: Number of recombinants, average recombination frequency and standard error (±SE) per marker interval.

No. of recombinants	GP34-77L	77L-IPM4c	IPM4c-77R	77R-IPM4a	IPM4a-111R	111R-73L	73L-218R	218R-PM5
S1-Cara	10 + 6 (*)	0	1	0	1	3	7	2
F1SH×RH	5	0	0	0	0	0	4	0
Recombination fraction	0.545	0 b	0.02	0 b	0.02	0.055	0.24	0.035
(± SE)	(±1.2) a		(±0.95) a		(±0.95) a	(±0.95) b	(±0.95) c	(±0.95) b

* Ten recombinants were found among 1,720 genotypes tested in the interval GP34-IPM3 (Bendahmane et al., 1997). Six recombinants were identified in the interval IPM3-77L among the 2,788 genotypes of S1-Cara. Recombination frequencies followed by a different letter differ significantly

Since there is only one recombinant found between *Gpa2* and *Rx* the average genetic distance between *Gpa2* and *Rx* is estimated to be 0.02 cM.

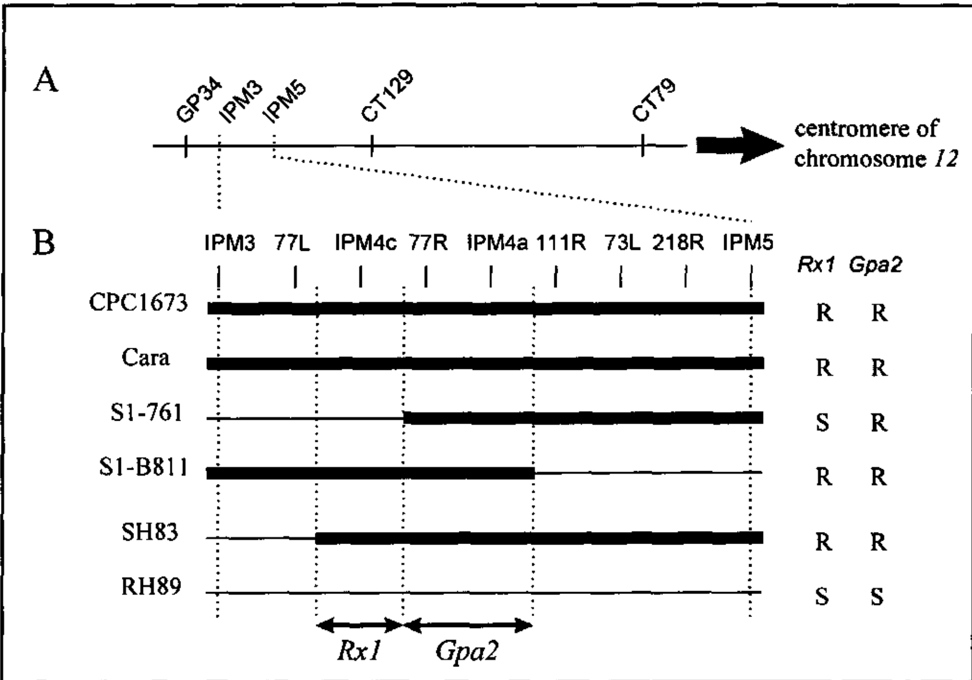

Figure 3. A: Relative postion of the Gpa2/Rx interval on potato chromosome 12. B: Graphical genotypes showing differences in the size of the CPC1673 derived segments. The presented genotypes border the respective intervals of Gpa2 and Rx. Introgression segments are indicated by thick bars. Size of marker intervals in B are not on scale.

DISCUSSION

The suggestion that both *Gpa2* and *Rx* may have originated from *S. tuberosum* ssp. *andigena* CPC1673 (Arntzen et al. 1994; Ritter et al. 1991) initiated the attempt to screen two different mapping populations for nematode and virus resistance. This attempt was further supported by the wealth of DNA markers available for the *Rx* region. At least two lines of evidence suggest that *Gpa2* and *Rx* derive from the same wild species accession. First of all, marker alleles linked to *Gpa2/Rx* of which the origin was confirmed in a progeny of selfings of the original CPC1673 clone, were identified in a number of PVX and *G. pallida* resistant cultivars harboring introgressions from CPC1673. Secondly, a reduced recombination frequency in the *Gpa2/Rx* region was observed. Only two recombinant plants were found in the two mapping populations analysed (total 3386 individuals) with cross-over events between markers 77L and 111R, a region spanning at least 150 kb (unpublished results). In contrast, increased recombination frequencies were

observed for the GP34-IPM3 interval, a region which spans approximately 100 kb and probably does not contain the introgression of CPC1673 in clone SH83. Such a reduced recombination frequency in the 77L-111R interval may be the result of a high degree of sequence polymorphism between the homologous chromosomes within this interval. This assumption is based on the model that sequence homology between homologous DNA strands initiates reciprocal genetic exchange during meiosis I (Borts and Haber, 1987; Priebe et al. 1994). Hence, a higher recombination frequency will be encountered in homozygous regions of common origin as compared to regions of heterogeneous origin (containing introgressions of foreign DNA). The suppressive effect of introgressions on recombination frequencies has been shown *e.g.* in barley (Görg et al. 1993) and in tomato (Rick, 1969; Liharska et al. 1996).

The tight linkage of both genes was determined after segregation analysis on a 3,386 offspring clones derived from two populations. For the IPM3-IPM5 region, 20 recombinant genotypes were found of which only one clone revealed a recombination event between *Gpa2* and *Rx*. Compared to this recombination fraction, the number of introgression segment length differences among the CPC1673 cultivars tested was remarkably high. The CPC1673 cultivars tested missed different parts of the IPM3-IPM5 region despite the fact that four out of five of these clones were probably selected on PVX resistance. This may be explained by the relatively early use of *S. tuberosum* ssp. *andigena* CPC 1673 in breeding programs which aimed to introgress resistance into cultivated *Solanum* species. The selection on agronomically important traits other than resistance may have severely reduced the fraction of CPC1673-derived DNA in concurrent hybrids. This explanation may also be applicable for the high level of allelic variation found at an RFLP locus near the *H1* gene (Niewöhner et al. 1995). It is noted that the recombination events in the IPM3-IPM5 interval occurred after introgression into cultivated potato because the cultivars which had the same selfed CPC1673 clone in their pedigree (*e.g.* cvs. Multa and Saturna from CPC1673-1; Alcmaria and Amaryl from CPC1673-20) contrained introgression segments of different lengths. The finding that a considerable level of variation exists between the sizes of the introgressed segments provides an additional source of 'rare recombinants' within a selected region of wild origin.

Studies on *Rx* resistance have shown that it can be considered mechanistically different from HR-types of plant resistance responses. The *Rx*-mediated response is not associated with a localized necrosis at the site of infection (Adams et al. 1986; Köhm et al. 1993) but mediates a specific inhibition of virus replication after being triggered by the coat protein of PVX (the elicitor of *Rx* resistance). The broad effectiveness of the *Rx*-mediated resistance response was demonstrated by co-inoculation of PVX and tobacco mosaic virus (TMV) (Bendahmane et al. 1995) which showed inhibition of both PVX and TMV replication in *Rx* protoplasts. Since non-specific degradation of host RNAs could be excluded from these experiments, it has been hypothesized that the *Rx* response may be directed towards a virus specific factor. A common feature in virus and nematode pathogenesis is that both pathogens are able to interfere with the plant cell metabolism. Cyst nematode juveniles penetrate the roots and migrate to the vascular cylinder where they initiate the redifferentiation of a procambial cell into a feeding site (Williamson and Hussey, 1996). Although the mechanism of *Gpa2*-mediated resistance has not yet been

thoroughly investigated, the severe reduction in cyst number and the poor egg contents of cysts recovered from resistant plants could indicate that the nutrient flow through the nematode feeding site is reduced in *Gpa2* plants. If *Rx* and *Gpa2* are related genes then it is intriguing to find out whether the poor feeding site development in the *Gpa2*-resistance response is due to either an *Rx* like response or to localized necrosis near or at the syncitium as in the case of *e.g.* the *H1* gene (Rice et al. 1985).

The identification of two *R*-genes which originate from a single wild species clone is very fortunate since the original *S. tuberosum* ssp. *andigena* CPC1673 clone has neither be selected for *Gpa2* nor for *Rx*. Clone *S. tuberosum* ssp. *andigena* CPC 1673 was collected in the late forties in the Andes region in South America in search for nematode resistance (Ellenby, 1952). Only five out of more than 1200 lines belonging to more than 60 species, harbored nematode resistance. The resistance in CPC1673 appeared to be conferred by a single gene *H* (later called the *H1* gene) which was discovered by screening populations derived from selfings of the original CPC1673 clone (Toxopeus and Huijsman, 1952; Huijsman, 1955). *H1* confers resistance to *G. rostochiensis* for which a gene-for-gene relationship has been established (Janssen et al. 1991), and the gene has been mapped on chromosome *5* (Gebhardt et al. 1993; Pineda et al. 1993). The occurrence of a second nematode resistance gene at an unlinked position on the genome of one and the same wild species clone may indicate that both *H1* and *Gpa2* originate from a common ancestor like the *L6* and *M* genes in flax (Anderson et al. 1997).

ACKNOWLEDGMENTS

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Summary and concluding remarks

This thesis describes genetic mapping strategies for the genomes of the potato cyst nematode and potato. Mapping in cyst nematodes was achieved by AFLP genotyping of single cysts and subsequent segregation analysis in a family of sibling populations. The genetic map of *Globodera rostochiensis* comprises nine linkage groups, a number similar to the haploid chromosome number determined. The low kb/cM ratio suggests that map based cloning of (a)virulence genes may be feasible for this organism.

For potato, a mapping strategy based on catalogued chromosome-specific AFLP markers facilitated the mapping of the nematode resistance loci *Gpa2* and *Grp1*. *Gpa2* confers specific resistance to *G. pallida* and is tightly linked to virus resistance at chromosome *12*. Locus *Grp1* is identified by QTL mapping. It confers resistance to *G. rostochiensis* pathotype Ro5, partial resistance to *G. pallida* and maps on chromosome *5*.

In conclusion, potato cyst nematode populations can be categorized into groups sharing similar molecular and virulence [Folkertsma 1997b]. In potato, a number of loci confer resistance to one or a few of these virulence groups. Nematode resistance loci are localized at genomic regions harboring other resistance factors known to be involved in gene-for-gene relationships. The co-localization of resistance loci indicate a structural similarity between the genes [Baker et al. 1997]. It is therefore tempting to speculate that gene-for-gene relationships determine the specificity of the interactions between potato cyst nematodes and potato.

Assessment of virulence of PCN populations: Potato cyst nematodes are endemic in the Andean region of South-America where they coevolved with their Solanaceous hosts. They are thought to be introduced into Europe relatively recently, after 1850, together with collections of potato species which were imported for breeding purposes. Only a limited part of the genetic variation present in their centre of origin has been introduced into Europe (Folkertsma 1997b). From the moment of their introduction onwards, the genetic variation in virulence within and between European populations has been determined predominantly by 1) the genetic structure of the primary founders, 2) random genetic drift and 3) gene flow. Mutation and selection can be excluded as a driving force for the observed variation; the species produce only one generation in a growing season, their multiplication rate is low, the time between generations is 2 to 4 years in a normal crop rotation and the active spread of the nematode is limited to several centimeters in the soil. It seems therefore highly unlikely that potato cyst nematode populations have

acquired other virulence characteristics than those already present at the moment of their introduction into Europe. Because virulence loci are selectively neutral in the absence of selection pressure by host resistance it has been argued that on the basis of the aforementioned features, the inter- and intraspecific variation of potato cyst nematode populations assessed by molecular characters which are randomly distributed over the genome are informative for the extent of the introduced variation in virulence.

Several molecular techniques have been applied to study the genetic diversity of potato cyst nematode populations. In this respect the three most informative techniques, two dimensional gel electrophoresis of proteins (2-DGE), RAPDs and AFLPs indicate that *G. rostochiensis* and *G. pallida* diverged millions of years ago (Bakker et al. 1992; Folkertsma et al. 1994; Folkertsma et al. 1996). This suggests that the virulence attributes of both species will be different.

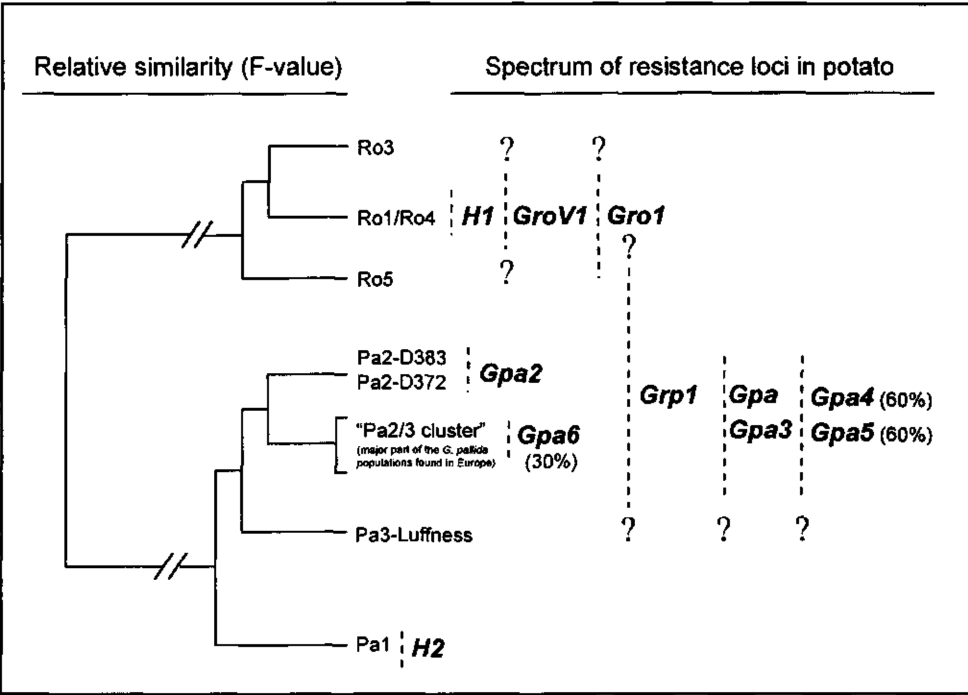

Figure 1: *Relative similarities of grouped potato cyst nematode populations found in Europe (Folkertsma et al. unpublished) and matching resistance factors in potato.*

Analysis of molecular variation between *G. rostochiensis* populations reveals three clusters of populations. These clusters can also be discriminated on the basis of virulence characteristics and are therefore indicated as Ro1, Ro3 and Ro5 clusters (Figure 1; from Folkertsma 1997b). The intraspecific variation of *G. pallida* populations shows a less clear cut separation. The number of polymorphisms (as determined by RAPDs and AFLP) seems to be larger than for *G. rostochiensis* but the differences between the majority of the populations seems to be less pronounced. Only a few (groups of) populations are clearly discriminated on the molecular level. Also these populations are

characterized by specific virulence attributes; one group is pathotyped Pa1, one group consists of the populations D383 and D372 (**chapter 6**) and one population exhibits an extremely virulent phenotype (population "Luffness"; Figure 1). For the majority of populations in the "Pa2/Pa3 cluster", a continuous range of variation has been found (Folkertsma 1997b). The genetic structure of these populations can be explained from bottleneck effects as a result of low initial population densities and subsequent random genetic drift (Folkertsma 1997b). The populations within this cluster presumably also originate from one or a few similar introductions and express therefore related virulence characteristics (indicated by Pa2/Pa3).

Identification of resistance loci to potato cyst nematodes in potato: Well characterized potato cyst nematode populations have greatly facilitated the identification of resistance loci in potato (Arntzen et al. 1994; **chapters 6 and 7**; unpublished). Combined with readily available QTL methods (Van Ooijen and Maliepaard, 1996ab) and the mapping strategy based on common AFLPs (**chapters 3, 4 and 5**), significant progress has been made during the last two years on the localization of genetic factors involved in PCN resistance (Table 1). The spectra of these resistance loci have shed a new light on the virulence characteristics of the populations within the Pa2/Pa3 cluster.

Mapping of *Grp1* revealed one major locus that confers, besides the resistance to *G. rostochiensis* pathotype Ro5, partially resistance to *G. pallida* population Pa3-Rook. At the time of publication of **chapter 7** we were unaware of the number of additional loci involved in the resistance of potato genotype 3778-16. In a recent study, locus *Gpa5* has been mapped which expresses *G. pallida* resistance which is comparable to *Grp1* (unpublished). The same resistant potato parent harbors locus *Gpa6*, which combined with *Gpa5*, confers almost complete resistance to Pa3-Rook (90% explained variance). It is noted that the specificity of *Gpa6* is directed to Pa3-Rook and not to Pa2-D383.

Table 1: Potato cyst nematode resistance loci in potato mapped by a strategy based on identification of common AFLP markers. G. ros = *G. rostochiensis*, G. pal = *G. pallida*, Chr. nr = chromosome number.

Locus	Pathogen	Chr. nr.	Solanum species used for mapping	Genotype nr.	Ref.
H1	*G. ros*	*5*	*S. tuberosum*	SH83-92-488	1, 2, chapter 6
Grp1	*G. ros & G. pal*	*5*	*S. tuberosum*	3778-16	chapter 7
Gpa2	*G. pal*	*12*	*S. tuberosum*	SH83-92-488	chapter 6, 8
Gpa3	*G. pal*	*11*	*S. tarijense*		P. Wolters, unpublished
Gpa5	*G. pal*	*5*	*S. tuberosum*	3704-76	unpublished
Gpa6	*G. pal*	*9*	*S. tuberosum*	3704-76	unpublished

1) Gebhardt et al. 1993;

2) Pineda et al. 1993

The virulence of population Pa3-Rook seems to be representative for the populations in the Pa2/Pa3-cluster (Folkertsma 1997b). In a parallel study, P. Wolters (CPRO-DLO) has determined the resistance spectra for locus *Gpa* (Kreike et al. 1994) and *Gpa3* (derived from *S. tarijense*; P. Wolters unpublished). Both *Gpa* and *Gpa3* confer resistance to ten populations which are representative for the Pa2/Pa3-cluster. In addition, *Gpa4* originating from *S. sparsipillum* has been identified which bestows the same level of resistance to Pa3-Rook as *Grp1* and *Gpa5*.

The partial resistance of *Grp1*, *Gpa4*, *Gpa5* and *Gpa6* may be explained by the heterogeneity on (a)virulence loci present in Pa2/Pa3 populations. In case different (a)virulence alleles are fluctuating in a population, a single *R* gene operating on the basis of a gene-for-gene relationship will confer partial resistance against the population as a whole whereas at the level of the individual, the *R* gene will confer absolute resistance against the matching avirulent genotype. In view of the gene-for-gene hypothesis this indicate that the virulence of the Pa2/Pa3 populations is determined by a only a few avirulence factors which can be matched by a similar number of resistance loci.

PCN resistance loci map to resistance gene clusters in potato: Genetic studies on many gene-for-gene plant-pathogen combinations suggest that the map positions of resistance loci are restricted to only a few genomic regions (reviewed in Baker et al. 1997). Within a plant species, these selected map regions harbor genes which often determine resistance to taxonomically unrelated pathogens (*e.g.* **chapter 8**). Nearly 20 resistance genes from various species have now been cloned and with the exception of two (*Hm1* and *mlo*; Johal and Briggs, 1992; Büsches et al. 1997), these genes seem to encode members of a signal transduction pathway. The (putative) gene products share a combination of structural motifs which are characterized as 1) a nucleotide binding site, 2) a leucine rich region, 3) a leucine zipper and 4) a protein kinase domain. On the basis of the structural similarity of the genes, it is hypothesized that resistance genes are evolutionary related components of a recognition system and that the mechanisms generating variation are entailed with a clustered distribution on the genome. Southern blots using cloned resistance genes as a probe showed the abundant presence of homologous sequences in many plant genomes. The map positions of these sequences coincide with regions where resistance to various pathogen species have been localized (Leister et al. 1996).

The potato map containing the genomic positions of the resistance gene like sequences has been complemented with the recently mapped PCN resistance loci (Figure 2). This map shows that all known potato cyst nematode resistance loci map to regions where other resistance specificities have been mapped. On the co-localization of the loci *Gpa2* and *Grp1* with other resistance loci was reported in **chapters 6 to 8**. Additionally, *Gpa3* resides in a region harboring resistance to *Meloidogyne chitwoodi* and *M. fallax* (*Rmc1*; Brown et al. 1995) and potato virus Y (Hämäläinen et al. 1996; Brigneti et al. 1996), *Gpa5* is located on the *Grp1* region, *Gpa6* maps on a region where in the homeologous tomato genome resistance to tomato spotted wilt virus has been mapped (unpublished). The precise location of *Gpa4* on chromosome *5* is unknown (P. Wolters, unpublished).

Towards characterization of genes which determine the potato cyst nematode-potato interaction: The cloning of these potato resistance genes benefits from the availability of elaborate genetic methods to dissect plant genomes. For potato, large diploid segregating populations can be constructed. The species is highly heterozygous and combined with informative marker techniques like AFLP and CAPS, the potato genome is accessible for cloning of genes on the basis of their map position. Methods to saturate the potato genome with molecular markers are described in **chapters 3, 4 and 5**.

When dense genetic maps are constructed, the markers can serve as starting points to perform a "walk" to the gene of interest or ultimately "land" on the gene itself (Tanksley et al. 1995). Molecular markers which flank a targeted genomic sequence can be used to screen genomic libraries to identify DNA fragments derived from that region. The additional information on DNA sequences provide then the ability to develop new markers and to screen the library again until the region is covered with overlapping clones. This procedure is followed in **chapter 8** in which bacterial artificial chromosomes (BACs) were used to identify new markers in the *Gpa2* region.

Map based cloning in potato cyst nematodes is less straightforward. The species is microscopic in size, non-inbred and has a long generation time. For the purpose of linkage mapping, we started by investigating chromosome behavior during meiosis. The chromosome number could readily be determined and no aberrant features during meiosis were observed (**chapter 1**). For construction of a genetic map, a linkage analysis procedure has been developed based on the analysis of sibling populations. Combined with the AFLP assay performed on single cysts a map has been constructed which covers about 65% of the genome (**chapter 2**). On the *G. rostochiensis* map, a random distribution of markers was encountered and estimates on the physical and genetic size of the genome revealed a low kilobase/centimorgan ratio. These attributes together with a mapping system for cyst nematodes has opened the way for map based cloning in this species.

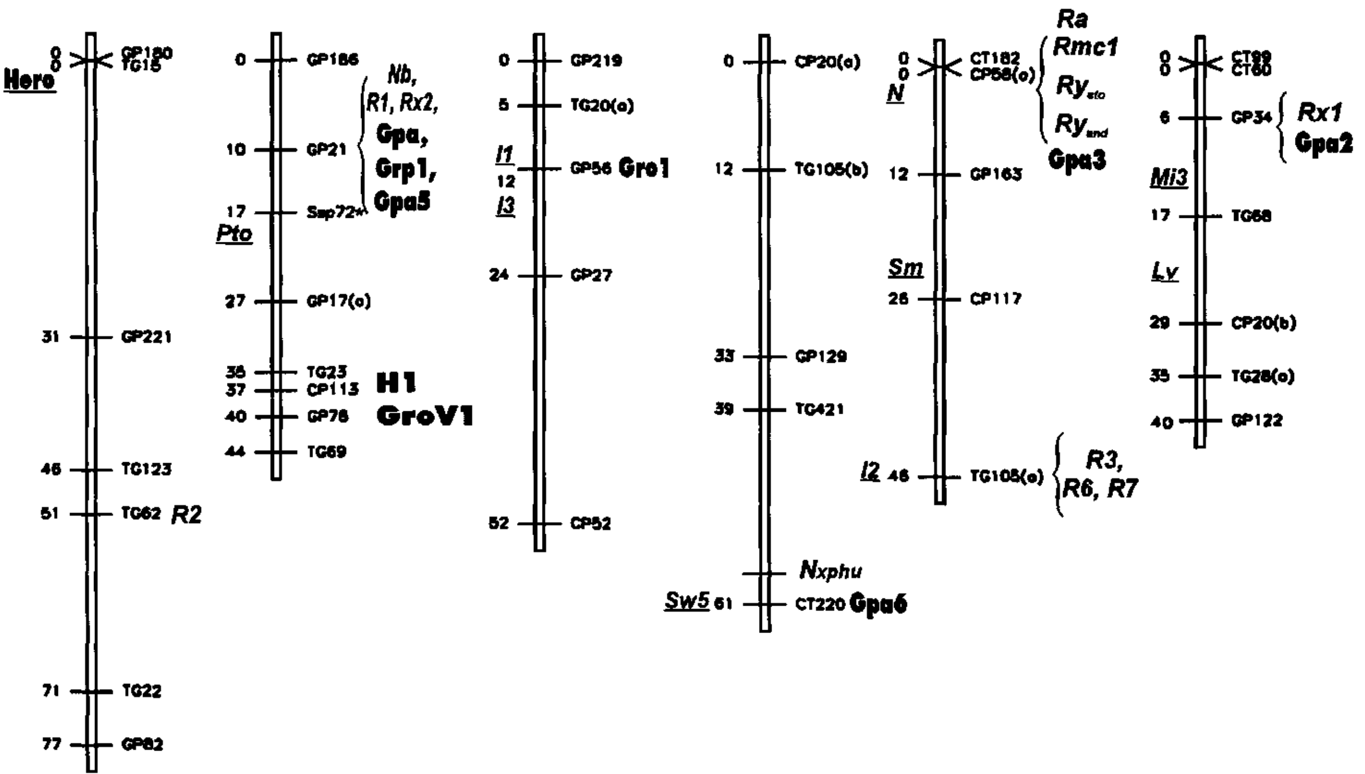

Figure 2: Map locations of resistance loci against potato cyst nematodes relative to other mapped resistance loci in potato and the related species tomato. Potato resistance loci are indicated right to the chromosomes in which PCN resistance loci are depicted in bold type. Resistance loci mapped in tomato are underlined and indicated to the left of a chromosome. Anchor RFLP marker order and distances are derived from Gebhardt et al. (1991) except for the overlapping marker locus GP129 and the loci TG421 and CT220 on chromosome 9 (Tanksley et al. 1992). These marker loci are chosen to facilitate the comparison with the map published by Leister at al. (1996), which harbors the map locations of 32 resistance gene-like sequences. The map locations of potato and tomato resistance loci are summarized in Leister et al. (1996). In addition, the genomic positions of loci conferring resistance to potato viruses Nb (de Jong et al. 1997), Nxphu (Tommiska et al. Theor. Appl. Genet. (1998) 96:840-843), Ra (Hämäläinen et al. Theor. Appl. Genet. (1998) 96:1036-1043) as well as to root knot nematodes Rmc1 (Brown et al. 1996) are added. It is noted that the regions of a QTL for PCN resistance on chromosome 4 (Bradshaw et al. (1998) 97:202-210) and Gpa4 on chromosome 5 are unknown. The Hero gene conferring resistance to G. rostochiensis in tomato is mapped on chromosome 4.

Conclusion and future prospects: The correspondence of the map position of nematode resistance loci with already mapped resistance gene clusters in potato indicates that the genes involved are members of known classes of resistance genes. For most of the cloned *R* genes it is known that they are involved in a gene-for-gene relationship. It is therefore tempting to speculate that these nematode resistance genes in potato emerged after gene-for-gene coevolution with the potato cyst nematode. This speculation is corroborated by the observation that the specificities of different resistance loci match with genetically defined clusters of populations (Figure 1). The correspondent grouping of potato cyst nematode populations based on molecular and virulence characteristics has been predicted by the gene-pool similarity concept (Bakker et al. 1993).

The matching of known resistance factors with the majority of the virulence types of potato cyst nematodes (Figure 1) offers ample opportunities for development of resistant potato cultivars. A consequence of the biology of potato cyst nematodes is that they have poor resistance breaking capabilities. This is illustrated by the observed disappearance of *G. rostochiensis* populations in Great Britain after growing of the *H1* gene. Since no new virulent types of *G. rostochiensis* emerged after growing of this resistance gene it appears that only *G. rostochiensis* populations of the pathotype Ro_1 have been introduced into this area (Trudgill 1991). Unfortunately, as observed for other potato growing areas in Europe, *G. pallida* populations came up in most areas where the *G. rostochiensis* populations disappeared. The emergence of *G. pallida* is explained by the latent presence of the species which has remained unnoticed before. Breeders are now focusing on the identification and introgression of resistance factors against *G. pallida*. The predictiveness of the virulence characteristics of PCN populations on resistance genes identified so far enables them to follow a more directed approach in the selection of clones with PCN resistance. Gene cloning strategies in potato are additional means to transfer natural resistance to commercial cultivars. The application of these technologies on natural resistance genes may provide a socially accepted means to short cut the time consuming process of breeding for resistance.

On the role of avirulence proteins acting as a ligand for triggering the resistance response by signaling via the product of a plant resistance gene has been hypothesized. Only in case of AvrPto from the bacterial pathogen *Pseudomonas syringae* pv. *glycinea* with Pto from tomato, direct binding has been demonstrated. For the products of all other avirulence genes (more than 30 avirulence genes have been cloned from bacterial pathogens (Bonas and Van den Ackerveken 1997); six from plant pathogenic fungi (reviewed in Knogge 1996) and two from viral pathogens (Culver and Dawson, 1991; Bendahmane et al. 1995) hardly anything is known about their function and interaction with the products of a plant gene. Binding studies between the Avr9 protein from *Cladosporium fulvum* and resistance gene product Cf9 from tomato indicate that the receptor-ligand model is far from complete (Kooman-Gersman et al. 1996; Kooman-Gersman, 1998). Insights into the molecular basis of the plant - pathogen interaction can only be achieved when all (interacting) partners are known. The ability to construct a genetic map for a plant parasitic nematode hopefully reinforces the attempts to characterize avirulence gene(s) from this pathogen. In this respect, the recent cloning of a likely pathogenicity factor from cyst nematodes (Smant et al. 1998) may be considered as

a breakthrough. The cloning of avirulence genes from the potato cyst nematode in concert with the isolation of resistance genes in potato will ultimately lead to a more profound understanding in the molecular basis of the interaction.

Nederlandse samenvatting

Deze samenvatting is geschreven voor niet-ingewijden in de biologie. Voor een uitgebreidere samenvatting van dit proefschrift wordt verwezen naar de "abstracts" die ieder hoofdstuk voorafgaan en naar de "Summary and concluding remarks".

Doel van het onderzoek: Dit proefschrift beschrijft een genetische studie naar de interactie tussen het aardappelcysteaaltje en haar gastheer aardappel. Inzicht in deze interactie is van belang omdat aardappelcysteaaltjes een grote plaag vormen in de aardappelteelt. In dit proefschrift komt de vraag aan de orde hoe factoren die de interactie tussen het aardappelcysteaaltje en de aardappel bepalen, geïdentificeerd kunnen worden. De identificatie van (a)virulentie- en resistentiefactoren is van cruciaal belang om meer inzicht te verkrijgen in de moleculaire mechanismen die betrokken zijn in de interactie tussen de plant en het pathogeen.

Omdat vrijwel niets bekend was van de biochemie die ten grondslag ligt aan de aardappelcysteaaltjes / aardappel interactie is in het hier beschreven onderzoek de vererving van eigenschappen bestudeerd die verantwoordelijk zijn voor de resistentie van aardappelplanten tegen aaltjes. Verder is een methode ontwikkeld die genetisch onderzoek mogelijk maakt naar (a)virulentiefactoren van het aaltje. Het onderzoek naar de aaltjes vereiste een heel andere aanpak dan het onderzoek naar het gewas aardappel. Daarom bestaat dit proefschrift uit twee delen. Het eerste deel (Hoofdstukken 1 en 2) heeft betrekking op de ontwikkeling van een methode waarmee in de toekomst (a)virulentiegenen van het aardappelcysteaaltje gekarakteriseerd kunnen worden. In het tweede deel (Hoofdstukken 3 t/m 7) wordt ingegaan op het in kaart brengen van resistentiegenen in de aardappel. Vervolgens (Hoofdstuk 8) worden de eerste stappen op weg naar de isolering van één van deze genen beschreven. Tenslotte ("Summary and concluding remarks") wordt een verband gelegd tussen de onderzoeksresultaten en een algemeen geldend model voor plant - pathogeen relaties.

Aardappelcysteaaltjes: Nematoden zijn rondwormen; een zeer grote en diverse groep van diersoorten waaronder ook de aardappelcysteaaltjes vallen. Aardappelcysteaaltjes zijn

plantparasitaire nematoden. In de loop van de evolutie hebben zij zich verregaand aangepast aan hun gastheer de aardappel en enkele aan de aardappel gerelateerde soorten (zoals de tomaat). Deze aanpassing heeft ertoe geleid dat het aaltje uitstekend is toegerust om voedsel aan de plant te onttrekken. Er zijn twee soorten van het aardappelcysteaaltje bekend: *Globodera rostochiensis* en *G. pallida*. Zij zijn de veroorzakers van de ziekte "aardappelmoeheid". De ziekte manifesteert zich door verstoring van de knolontwikkeling, het verwelken en uiteindelijk afsterven van de plant. Wereldwijd worden opbrengstverliezen als gevolg van besmetting met het aardappelcysteaaltje geschat tussen 5 en 10%.

Het effect van besmetting met aardappelcysteaaltjes is tweeledig. Ten eerste is er het genoemde effect van infecties van de plant. Daarnaast stelt de persistentie van de cysten in de bodem akkerbouwers voor grote problemen. De cysten laten zich het best omschrijven als een verharde zak met eieren (afgebeeld in Figuur 1 van Hoofdstuk 8). Op deze wijze kunnen aardappelcysteaaltjes soms jaren zonder waardplant in de bodem overleven. Alleen zeer milieubelastende chemicaliën zijn in staat de eieren in de cyst te doden. Het gebruik van deze bestrijdingsmiddelen is slechts nog in beperkte mate toegestaan. Daarom vallen akkerbouwers voor de bestrijding van aardappelmoeheid terug op vruchtwisseling en het gebruik van resistente aardappelrassen. Vruchtwisseling is vanuit economisch oogpunt weinig aantrekkelijk omdat een groot deel van de inkomsten in de akkerbouw door de teelt van aardappelen gegenereerd wordt. De verwachting is dan ook dat het aandeel van resistente rassen in de aardappelteelt in de toekomst nog zal toenemen.

Resistentie in aardappel / (a)virulentie in aardappelcysteaaltjes: Voor het optreden van resistentie in een plant ofwel (a)virulentie in een plantenpathogeen is in de jaren '50 een hypothese opgesteld die bekend staat als de gen-om-gen relatie. Deze hypothese stelt dat voor ieder resistentiegen in de plant er een corresponderend avirulentiegen in het pathogeen aanwezig is. Deze relatie is zeer specifiek. Indien het pathogeen in staat is het avirulentiegen te veranderen dan is de plant niet meer in staat het pathogeen als indringer te herkennen en zal het pathogeen zich kunnen vermeerderen. In moleculaire termen wordt voorgesteld dat de producten van resistentiegenen een soort chemische sensoren zijn die, zodra het avirulentiegenproduct herkend wordt, een signaal aan de plantencel doorgeven om zich te beschermen tegen de indringer. Het stelsel van afweerreacties dat planten in staat stelt zich tegen indringers te beschermen lijkt in zowel resistente als vatbare planten aanwezig. Volgens de gen-om-gen hypothese wordt echter de herkenning van het pathogeen, dat de afweerreactie initieert, bepaald door het product van één enkel gen.

Genetische kaarten: De vererVingsstudies die in dit proefschrift zijn beschreven zijn uitgevoerd met behulp van genetische kaarten. Genetische kaarten geven schematisch weer hoe verschillende kenmerken ten opzichte van elkaar op de chromosomen gelokaliseerd zijn. Kenmerken die gebruikt worden voor genetisch onderzoek kunnen zichtbaar zijn zoals bloemkleur, knolgrootte en resistenties maar ook abstract zoals DNA

markers. DNA markers zijn korte DNA fragmentjes die met behulp van biochemische detectiemethoden worden geïdentificeerd. In hoofdstuk 2 tot en met 8 komen verschillende DNA markers aan de orde waarvan de AFLP marker de belangrijkste is. Het aantal DNA kenmerken dat met behulp van de AFLP techniek geïdentificeerd kan worden is enorm. Hiermee is het mogelijk om vast te stellen welke kenmerken in een nakomelingschap van de vader of van de moeder afkomstig zijn. Verder kunnen met behulp van DNA markers chromosomale regio's afgebakend worden. Hierdoor kan een verband gelegd worden tussen het voorkomen van een eigenschap (bv. resistentie) en de aanwezigheid van bepaalde DNA markers. Tenslotte kunnen DNA markers gebruikt worden om genen te kloneren. Door een geselecteerde chromosomale regio te verzadigen met DNA markers is zeer nauwkeurig vast te stellen waar een bepaald gen ligt. De flankerende markers aan dat gen kunnen daarna als een soort index gebruikt worden om in een bibliotheek van DNA fragmenten het gen op te sporen.

Inhoud van dit proefschrift: In dit proefschrift worden de ontwikkeling en de toepassing van genetische kaarten beschreven in het onderzoek naar virulentie- en resistentiefactoren die de interactie tussen aardappelcysteaaltjes en de aardappel bepalen. In de eerste twee hoofdstukken komt het aaltje aan bod. Weinig kennis was beschikbaar over de genetica van het aardappelcysteaaltje. Daarom is begonnen met fundamenteel werk aan de chromosomen van het aaltje. Het chromosoomaantal van het aardappelcysteaaltje is bepaald en enig inzicht is verkregen in het gedrag van chromosomen tijdens de celdeling (**hoofdstuk 1**). Deze studie gaf geen aanleiding om te twijfelen aan de haalbaarheid van het opstellen van een genetische kaart van aaltjeschromosomen. Echter, de biologie van het aaltje is dermate complex dat dit de constructie van de genetische kaart in de weg zou kunnen staan. In **hoofdstuk 2** is een methode beschreven waarin met behulp van enkele aanpassingen op conventionele karteringsstrategieën toch een kaart van het aaltjesgenoom kon worden opgesteld.

Voor de aardappel lag de beginsituatie van het onderzoek totaal anders dan voor het genetisch onderzoek naar aaltjes. Een gedetailleerde chromosoomkaart van het aardappelgenoom was al voorhanden en bovendien was er in Wageningen een collectie aardappelmateriaal beschikbaar waarin verschillende resistenties tegen aardappelmoeheid waren ingekruist. Minder goed ontwikkeld waren methoden waarmee chromosomale regio's met elkaar vergeleken konden worden. Hierdoor was het noodzakelijk om voor ieder nakomelingschap waarin een bepaalde eigenschap (bv. resistentie) vererfde, chromosoom voor chromosoom na te lopen alvorens de locatie van die eigenschap kon worden vastgesteld. Met de ontwikkeling van de AFLP techniek is daar verandering in gekomen. De AFLP techniek maakt het mogelijk om de vererving van een groot aantal kenmerken tegelijk te volgen. Dit heeft ertoe geleid dat de bestaande genetische kaart van aardappel in zeer korte tijd kon worden uitgebreid met AFLP markers (**hoofdstuk 3**). Vervolgens zijn verschillende genetische kaarten van aardappel gemaakt en is een methode ontwikkeld waarmee met behulp van AFLP markers deze kaarten met elkaar vergeleken kunnen worden (**hoofdstuk 4**). Voor de vergelijking van genetische kaarten bleek het zeer praktisch te zijn om dit via een naslagwerk van chromosoom-specifieke

AFLP markers te laten verlopen (**hoofdstuk 5**). Dit naslagwerk van AFLP markers is gebruikt om resistentiegenen tegen aardappelcysteaaltjes op de kaart van de aardappel te plaatsen. In **hoofdstuk 6** is de lokalisatie van een resistentiegen tegen *G. pallida* beschreven. Analoog hieraan is een complex vererving resistentie tegen aardappelcysteaaltjes op de kaart van de aardappel gezet (**hoofdstuk 7**). De fijnkartering van het in hoofdstuk 6 beschreven gen in **hoofdstuk 8** uiteengezet. Tenslotte worden in de **summary and concluding remarks** de resultaten van de laatste drie hoofdstukken geïntegreerd met het promotieonderzoek van Rolf Folkertsma (1997b). Op basis van onze bevindingen en nog niet gepubliceerd onderzoek menen we sterke aanwijzingen te hebben dat gen-om-gen relaties een belangrijke rol spelen in de coevolutie van het aardappelcysteaaltje en aardappel.

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Nawoord

Graag wil ik mij richten tot eenieder die een bijdrage heeft geleverd aan het tot stand komen van dit proefschrift. Meer lieden dan de schrijver dezes verdienen het om op de omslag vermeld te worden. Daarom zal ik trachten hen op deze plaats toch wat van die eer te doen toekomen.

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Curriculum vitae

Jeroen Rouppe van der Voort wordt geboren op 22 augustus 1967 te Breda. Hij doorloopt het H.A.V.O. en het V.W.O. op het Onze Lieve Vrouwe Lyceum te Breda waarna hij zijn militaire dienstplicht vervult als onderofficier bij de artillerie. In 1987 vangt hij aan met de studie Biologie aan de Universiteit van Amsterdam. Hij kiest voor de afstudeerrichting moleculaire genetica en verricht zijn doctoraalonderzoeken achtereenvolgens bij de vakgroep Systematiek en Evolutie van de Universiteit van Amsterdam (dr. ir. A. van Heusden en prof. dr. K. Bachmann), het Laboratorium voor Monoklonale Antistoffen te Wageningen (drs. E. Beerling en dr. ir. A. Schots) en de vakgroep Nematologie van de Landbouw Universiteit Wageningen (dr. J. Roosien en dr. ir. J. Bakker). In 1991 wordt hem de Unilever Research Prijs toegekend. Van 1992 tot en met 1996 is hij assistent in opleiding bij de vakgroep Nematologie. Sinds 1997 is hij als onderzoeker werkzaam bij dezelfde vakgroep in dienst van de Nederlandse Organisatie voor Wetenschappelijk onderzoek.