Genetic analyses of root-knot nematode resistance in potato

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Chapter 1

General Introduction

Potato

The potato (*Solanum tuberosum* L. ssp. *tuberosum*) originates from the Andes in South America and has been distributed globally after its introduction in Europe around 1570 (Hawkes 1994). Potato is nowadays the most important non-cereal crop in the world and combines a high (potential) yield with a high nutritional value. The potential yield is often not reached due to biotic and non-biotic stresses (Oerke *et al*. 1994). Ranking fourth in food production with 311 million tons per year (http://apps.fao.org/default.jsp), genetic improvement of potato by means of plant breeding may contribute greatly to the world's food supply. Those improvements can be attained by resistance and tolerance to stress conditions and mechanical damage and by resistance and tolerance to the many diverse diseases and pests during growth and storage. Many resistance genes to the various diseases and pests are available in the broad gene pool of potato which contains some 235 species. Besides resistance, the *Solanum* species give access to various useful genes including tolerance to abiotic stress conditions like frost, heat and drought, and quality traits like enzymatic browning, dry matter content and protein composition and content.

The wild species used until now in potato breeding are mainly selected for their resistance characteristics. A total of 266 pathogens and pests are able to affect the potato (Mendoza 1987), of which the oomycete *Phytophthora infestans* is the most devastating. This disease causes enormous losses and has lead to the well-known Great Famine in Ireland in 1845 and 1846. As from that moment potato breeders tried to breed for resistance to late blight, using resistant wild potato species (Hawkes 1990). The introduction of wild potato species, for resistance breeding against *P. infestans*, caused most likely also the introduction of another important pest in Western Europe: the potato cyst nematodes *Globodera rostochiensis* and *G. pallida*. Soon after the detection of this pathogen, potato breeders looked for resistance in wild species. The first reported resistance to *G. rostochiensis* was the *H1* gene from *S. tuberosum* ssp. *andigena* (Ellenby 1952). The successful introduction of resistance to *G. rostochiensis* is an example of the use of the genetic resources of potato. By extensive efforts in plant breeding, using wild species, a lot of cultivars resistant to *Globodera* spp. are nowadays available.

Most of the common cultivated potatoes are tetraploid with a somatic chromosome number of 2n=4x=48. The majority of the wild tuber-bearing *Solanum* species is however diploid (2n=2x=24). A breeding program of potato at the diploid level enables direct gene transfer from these wild diploid *Solanum* species to the tetraploid *S. tuberosum*. Such a diploid potato breeding program starts with the pollination of tetraploid potato cultivars with specific clones of *S. phureja* (2x), which results in the development of pseudogamous seeds (Hermsen & Verdenius 1973). The dihaploid clones derived from these crosses are the basis of the diploid breeding program. Subsequently, the dihaploid clones with the best agronomical performance are crossed with the wild diploid species. Crossing the clones with a high agronomic value with inferior but resistant wild species will mostly implicate a setback of performance of agronomic traits like tuber shape, length of stolons and eye depth. The resulting progeny of these crosses will also have an overall decreased quality, such as more discoloration after cooking or lower chipping quality. Several backcrosses will be necessary to return to an acceptable agronomic value. This backcrossing process is more efficient on the diploid level, due to the disomic

inheritance vs tetrasomic inheritance on the tetraploid level, resulting in a larger phenotypic variation. Diploid breeding also implicates that a smaller progeny size and a lower number of backcrosses is required.

After cycles of breeding at the diploid level, the tetraploid level can be regained via 2n gametes. Of the two types of 2n-gametes the First Division Restitution (FDR) is preferred above the Second Division Restitution (SDR) because of its positive effect on yield (Hutten *et al.* 1994). Because most of the 2n-pollen is generated via FDR, and because the selection for 2n-pollen is less laborious than the selection for 2n-eggs, the recovery of the tetraploid level is preferred by means of 4x.2x crosses.

Besides the direct gene transfer and more efficient breeding, the use of diploid potatoes also has a major advantage in genetic studies. The polyploid nature of potato makes genetic research very complicated, especially when more genes are involved in the studied character.

Nematodes

Nematodes belong to a large phylum of worm-like animals of which a large part is plant pathogenic. The most economically important nematode species in relation to potato are the potato cyst nematode *Globodera* spp. and the root-knot nematode *Meloidogyne* spp.

Until the late 60s most of the diseases and pests, like *P. infestans* and potato cyst nematodes, were treated with pesticides. Due to environmental concerns the use of chemicals should be minimised as much as possible. The restriction of the use of chemicals in agriculture raised the need for resistant crops. Potato breeders have been successful in developing potato cultivars with resistance to *Globodera* spp. This success resulted in a drastic reduction of the use of nematicides. The drawback of the reduced use of chemical soil disinfection is the rising problem nowadays with other plant pathogenic nematodes, amongst others the *Meloidogyne* spp.

Three species of *Meloidogyne* are important in potato: the Northern root-knot nematode *M. hapla*, the Columbian root-knot nematode *M. chitwoodi* and the nematode *M. fallax*, described as biologically and morphologically different from *M. chitwoodi* (Karssen 1996). *M. chitwoodi* has been found in the following states of the USA: California, Colorado, Idaho, New Mexico, Nevada, Oregon, Utah, Washington and Texas (Szalanski *et al.* 2001); in Argentine, Mexico, Portugal, South Africa, The Netherlands, Belgium (summarised in Van der Beek 1997) and in Germany (Müller *et al* 1996). Populations of *M. fallax* are more frequently detected after the first description and molecular methods became available to identify the species (Zijlstra 1997). *M. fallax* is currently present in The Netherlands (Karssen 1996), France (Daher *et al*. 1996) and New Zealand (Marshall *et al.* 2001). World-wide the Northern root-knot nematode *M. hapla* is more widespread than *M. chitwoodi* and *M. fallax.*

The root-knot nematodes are like the *Globodera* spp. endoparasitic and mainly sedentary in the roots. These specialised plant parasites have a highly developed interaction with their hosts. The second-stage juvenile (J2) is the infective stage of the *Meloidogyne* nematode. Hatching from the eggs the J2 will penetrate the root tip and moves intracellularly towards the meristematic region. At the meristematic region the J2 turns and moves towards the vascular

cylinder where it induces a feeding site and becomes sedentary. The root-knot nematodes modify plant cells into several adjacent giant cells, where the potato cyst nematode induces syncytial feeding sites. Both feeding sites have intense metabolic activity and function as transfer cells for solutes. The root-knot nematodes also induce the plant to produce galls, surrounding them. Unlike the *Globodera* spp., where the female body develops into a cyst containing the eggs, the *Meloidogyne* spp. deposit their eggs in egg-masses, a gelatinous matrix attached to the female. The root-knot nematodes hibernate as eggs or as juveniles.

In contrast to the *Globodera* spp. whose host range is confined to several *Solanum* species, the *Meloidogyne* spp. are able to multiply on many diverse hosts. The host range of *M. fallax* and *M. chitwoodi* is rather similar, containing numerous dicotyledonous plants such as sugar beet, carrot, black salsify, lettuce, pea, alfalfa and tomato, but also cereals and grasses. Maize is the most prominent differential in defining the host range of the two species. Maize is a poor host to *M. fallax* and a good host to *M. chitwoodi* (van Meggelen *et al.* 1994). In *M. chitwoodi* two different races are recognised based on the reproduction on carrot and alfalfa. Thor alfalfa is a poor host to race 1 and a good host to race 2, where Red Cored Chantenay carrot is a good host to race 1 but a poor host to race 2 (Santo & Pinkerton 1985). This classification based on carrot and alfalfa is not always unambiguous, as race 2 sometimes produces egg masses on the non-host carrot (Van der Beek *et al* 1999). Based on the differences in reproduction on *S. bulbocastanum* carrying the R_{Mc1} resistance gene, *M. chitwoodi* race 2 can be divided into two pathotypes, of which the virulent one was formerly designated as race 3 (Mojtahedi *et al.* 1994, 1998). The analysis of eight *M. chitwoodi* populations indicates that there is probably no race 2 present in The Netherlands (Van der Beek *et al*. 1999).

The host range of *M. hapla* is also very broad containing most dicotyledonous food crops, but this nematode species is not able to multiply on monocotyledons. Therefore, a crop rotation with monocotyledons is a feasible solution in order to control *M. hapla*. The inability of *M. hapla* to multiply on monocotyledons is most likely one of the reasons of its lower incidence on potato as compared with *M. chitwoodi*. Hilbrands Laboratorium voor Bodemziekten (pers. comm. J. Peltjes) observed less *M. hapla* infestations then *M. chitwoodi* infestations in the North-Eastern part of The Netherlands, an area with an intensive potato production. Another reason for the lower incidence of *M. hapla* on potato is the more rapid reproduction of *M. chitwoodi* at a lower soil temperature (O'Bannon & Santo 1984). In contrast to *M. chitwoodi* there are at present no host races described of *M. hapla*, but two cytological races of *M. hapla* are known: the most common meiotic parthenogenetic race A and the obligate mitotic parthenogenetic race B. Both cytological races are found in The Netherlands.

The symptoms caused by an infection of *M. chitwoodi* and *M. fallax* are similar. Tubers of infected plants show varying degrees of galling and the infestations give the tuber skin a rough appearance. Galls in freshly harvested potato tubers are translucent and difficult to detect, but after a few months the egg sacs turn brown and can be seen as brown spots in the flesh. The dark necrotic spots in the tuber flesh, the galled tubers and the rough skin turn the infected potato an unmarketable product. These typical symptoms are absent in tubers infected with *M. hapla*. The symptoms caused by this species in potato are restricted to losses in yield. A yield loss of 70% has been reported at a high level of infection with *M. hapla* (MacGuidwin & Rousse

1990). In addition, the multiplication of *M. hapla* during the growth of a susceptible potato will result in higher residual population densities and can lead to severe damage to the next crop in the rotation, like carrot, onion and black salsify.

The wide host range of *M. chitwoodi* and *M. fallax* makes crop rotation not a feasible solution to decrease the nematode population and to minimise yield losses. In order to minimise the chemical input in agriculture, the development of resistant plants offers an environmental friendly solution to nematode infestations. The current lack of resistance in the cultivated potato forces the breeder to using wild *Solanum* species. Resistance to *M. chitwoodi* and *M. hapla* was detected in *S. bulbocastanum* and *S. hougassii* (Brown *et al.* 1989, 1991). Because *S. bulbocastanum* could not be crossed with cultivated potato, somatic hybridisation was used between this wild diploid Mexican species and a tetraploid *S. tuberosum* in order to introgress the resistance in cultivated potato (Austin *et al*. 1993). Backcrosses of the somatic hybrid resulted in resistant clones, and subsequently genetic analysis led to the mapping of the monogenic resistance gene R_{Mc1} on chromosome 11 (Brown *et al* 1995, 1996).

The introgression of the resistance present in the hexaploid Mexican wild species *S. hougasii* was easier because this species was crossable with *S. tuberosum.* It is suggested that this wild species carries independent loci involved in resistance to both race 1 and race 2 of *M. chitwoodi* (Brown *et al.* 1999). Additionally a broad set of other wild *Solanum* species has been tested for resistance to *M. chitwoodi*, *M. fallax* and *M. hapla* (Janssen *et al.* 1996a, 1997b). Populations produced from intraspecific crosses of several wild *Solanum* species were tested to dissect the genetics of the resistance (Janssen *et al.* 1997a). First attempts were also made to introgress resistance into cultivated potato using the wild species *S. bulbocastanum*, *S. cardiophyllum*, *S. brachistotrichum*, *S. stoloniferum*, *S. fendleri* and *S. hougasii*, of which only the latter three crossings were successful (Janssen *et al.* 1997c).

A major aspect that could influence the durability of the resistance is the presence and allele frequency of (a-)virulent factors in the pathogen. Therefore, the strategy to exploit resistance genes against the root-knot nematodes should dependent on the variation in virulence in natural populations. Virulent individuals are able to reproduce on resistant host plants. Knowledge of the virulence factors in the nematode population is also helpful in the search for additional resistance genes. The current lack of resistance in cultivated potato explains why only a minor proportion of the variation in virulence of *M. chitwoodi*, *M. fallax* and *M. hapla* to the tested potato cultivars could be described (Van der Beek *et al.* 1998a). The presumed genetic factors causing the variation should, therefore, be very small. There was, however, a significant difference in aggressiveness between the tested nematode populations. Other studies using resistant wild *Solanum* spp. detected significant variation in virulence between and within the *Meloidogyne* spp.. *M. hapla* more often showed a difference in virulence as compared with *M. chitwoodi* and *M. fallax*. Although the virulence of the latter two species is very similar on the different tested *Solanum* spp. there is a difference in reproduction on *S. chacoense*. This wild *Solanum* species is a good host for *M. chitwoodi*, but rather resistant to *M. fallax* (Van der Beek *et al.* 1998b, Janssen *et al.* 1997b).

Van der Beek *et al.* (1998b) detected little variation in virulence to *S. chacoense* and *S. stoloniferum* among the tested *M. chitwoodi* populations. The variation in virulence within the *M.*

chitwoodi populations to the resistance in *S. bulbocastanum* has led to the nomination of race 3, later described as a virulent race 2 population (Motjahedi *et al.* 1994, 1998). Selection of *M. chitwoodi* on a resistant *S. fendleri* clone led to a virulent population of *M. chitwoodi* race 1 population (Janssen *et al.* 1998). Until now the amount of virulence factors present in *M. chitwoodi* is unclear and should be specified by testing a wide range of wild *Solanum* species and using the molecular data of the *M. chitwoodi* populations. Molecular analyses with two-dimensional gel electrophoresis (2-DGE) of different populations of *M. chitwoodi*, including the races 1 and 2 from the USA and the virulent race 2 population, did not show clear genetic differentiation between the populations (Van der Beek *et al.* 1997).

The results of 2-DGE of *M. hapla* show that the tested populations are rather heterogeneous as compared with *M. fallax* and *M. chitwoodi* (Van der Beek *et al.* 1997). Considerable variation in virulence within *M. hapla* populations was found to three wild *Solanum* species: *S. chacoense*, *S. hougasii* and *S. sparsipilum* (Van der Beek *et al.* 1998b). Although the genetic variation between the two cytological races of *M. hapla* was significant, no association could be found between the virulence and these races. Based on the estimated interaction between the *M. hapla* populations and the three *Solanum* spp. Van der Beek *et al.* (1998b) suggested that approximately five different virulence factors are present in the *M. hapla* populations.

Mapping of resistance genes

The developments and improvements in DNA marker technology has led to an increased understanding of genetics and has opened the possibility for an accurate connection of the genetic information with the phenotype. The diversity of molecular DNA markers available nowadays and the ongoing development in genomics offers the potato breeder a valuable tool for selection that should lead to an improvement of selection efficiency. The basic principle of marker assisted selection (MAS) is the selection of characters with the help of easily detectable phenotypes or DNA markers. Such a marker tightly linked to the gene of interest, will most likely be passed together with the character of interest to the next generation.

The advantages of MAS over phenotypic screening are evident in the case of characters expressed late in plant development or to characters that inherit recessively. The introgression of genes from wild species can be accelerated using MAS in the successive backcrosses. MAS can be used for the positive selection of the gene of interest and the recipient genome, and negative selection of the donor genome. MAS allows the selection for resistance without the need to use a resistance test. This is relevant when the pathogen is a quarantine organism and special precautionary measurements should be taken for the phenotypic screening.

Molecular markers can also be used to select for complex traits, such as yield or starch content. These complex traits are usually of a quantitative nature and strongly effected by the environment. By dissection of the trait into Mendelian loci, selection can be carried out at these so called quantitative trait loci (QTL loci), without looking at the phenotype and thus without the effects of the environment on the genotype. Especially in case of traits with a low heritability the use of molecular markers is a requisite.

Marker technologies

With the development of the AFLP technology (Vos *et al*. 1995) a powerful molecular marker technique was added to the existing techniques as RFLP, RAPD and SSR. The AFLP marker technology resulted in an easily achieved and high amount of markers and accelerated the genetic research. The large numbers of markers allowed the construction of a marker saturated linkage map of potato (Van Os 2005). Many genetic studies in potato make use of the AFLP technology nowadays, but also SSR and CAPS are used. The latter two locus specific marker technologies make use of sequence information for the construction of primers. One of the advantages of AFLP markers is that no sequence information is necessary. The AFLP technique is, however, too expensive to be used for a high throughput MAS. Therefore, the AFLP markers need to be transformed into less expensive markers. Recently, a new technique was published that simplifies the transformation of AFLP markers into CAPS or SNP markers (Brugmans *et al.* 2003). The most ideal marker for a breeder is a SNP based on the sequence information of the alleles of the gene of interest. The transformation of AFLP markers into SNPs makes the MAS more cost-effective and opens the opportunity to multiplex different traits for MAS.

Genetic map

The DNA markers as described above, will behave as genes and inherit in a Mendelian way. Co-segregation of the molecular markers allows the construction of a genetic map. In order to handle the huge amount of marker data, computer programmes are developed to estimate the recombination frequency between the markers and to order the markers. In this way the first genetic map of potato was constructed using 134 RFLP and isozyme markers (Bonierbale *et al*. 1988). Later the first genetic map of potato using AFLP markers was published, by adding 264 AFLP markers to 217 RFLP, isozyme and morphological trait loci (Van Eck *et al.* 1995). Recently an ultra high density map of a diploid potato population was constructed containing about 10,000 markers (http://potatodbase.dpw.wur.nl/UHDdata.html, van Os 2005). A large amount of these AFLP markers has been shown to be chromosome specific and can be detected by co-migration on the gel combined with a similar mapping position (Rouppe van der Voort *et al.* 1997a).

Molecular markers have proven to be useful in map-based gene cloning projects. After the construction of a high resolution map surrounding the gene of interest, the gene can be located in a BAC library by chromosome walking. For example the *R1* gene conferring resistance to *P. infestans* was mapped for the first time in 1992 (Leonards-Schippers *et al.* 1992), later this gene was fine-mapped (Meksem *et al.* 1995) and finally cloned (Ballvora *et al.* 2002). Other cloned resistance genes in potato are the *Gpa2* gene conferring resistance to *G. pallida* (Van der Vossen *et al.* 2000), the *Rx* gene conferring resistance to Potato virus X (Bendahmane *et al.* 1999), the genes *RB* (Song *et al.* 2003), *Rpi-blb1* (Van der Vossen *et al.* 2003) and *R3a* (Huang *et al.* 2005) conferring resistance to late blight.

Mapping strategy

Mapping strategy is depending on the inheritance of the trait. In case of a monogenic inheritance with a distinct segregation, the Bulked Segregant Analysis (BSA) (Michelmore *et al.* 1991) approach is a very effective way for the detection of linked markers, especially with the high amount of AFLP markers per primer combination found in potato. Only the putative linked markers found with the BSA will be tested on all individual genotypes of the mapping population. This will result in a small piece of the genetic map including the resistance gene. The BSA approach has shown its value in mapping studies using diploid populations (a.o. Hämäläinen *et al.* 1998, Tommiska *et al.* 1998) as well as tetraploid populations (a.o. Li *et al.* 1998). However, many important characters exhibit a continuous variation and are often controlled by multiple genes. Usually these characters are influenced by environmental factors, hampering a clear insight into the genetics of the character. In order to locate a QTL the construction of a genetic map of the complete genome is inevitable. The known mapping position of many AFLP markers in potato simplifies the construction of these maps. Many resistance genes, QTLs as well as major genes (M) have been localised in different published maps. In Table 1 resistance genes mapped so far are listed.

Table 1: Overview of resistance genes localised on the genetic map of potato.
Type M = monogenic qualitative gene; type QTL = quantitative gene.

Nematode resistance					
Name	**nematode**	**type**	**source**	**chromosome**	**reference**
H1	*G. rostochiensis* Ro1	M	adg CPC 1690	5	Pineda *et al.* 1993 Gebhardt *et al.* 1993
H3	*G. pallida*	QTL	adg CPC2775	4	Bradshaw et al., 1998
$Gro_{V.1}$	*G. rostochiensis* Ro1	M	vrn $(VT^n)^2$62-33-3	5	Jacobs *et al.* 1996
Gro_1	*G. rostochiensis* Ro1 Ro5	M	spg H80.696/4	7	Barone *et al.* 1990
$Gro_{1.2}$	*G. rostochiensis* Ro1	QTL	spg	10	Kreike *et al.* 1993
$Gro_{1.3}$	*G. rostochiensis* Ro1	QTL	spg	11	Kreike *et al.* 1993
$Gro_{1.4}$	*G. rostochiensis* Ro1	QTL	spg	3	Kreike *et al.* 1996
Grp1	*G.* spp. Ro5, Pa2, Pa3	QTL	78-3778	5	Rouppe van der Voort *et al.* 1998b
Gpa2	*G. pallida*	M	adg CPC 1673	12	Rouppe van der Voort *et al.* 1997b
Gpa3	*G. palida* Pa3	M	tar	11	Wolters *et al.* 1998
Gpa4	*G. palida* Pa2, Pa3	QTL	spl	5	Wolters *et al.* 1998
$GpaV^s_{spl}$	*G. palida* Pa2, Pa3	QTL	spl	5	Caromel *et al.* 2005
$GpaXI^s_{spl}$	*G. palida* Pa2, Pa3	QTL	spl	11	Caromel *et al.* 2005
Gpa	*G. pallida* Pa2, Pa3	QTL	spg	5	Kreike *et al.* 1994
Gpa	*G. palida* Pa2, Pa3	QTL	tbr	4	Bradshaw *et al.* 1998
Gpa5	*G. pallida* Pa2, Pa3	QTL	3704-76	5	Rouppe van der Voort *et al.* 2000
Gpa6	*G. pallida* Pa3	QTL	3704-76	9	Rouppe van der Voort *et al.* 2000
GpaM1	*G. pallida*	QTL	spg	5	Caromel *et al.* 2003
GpaM2	*G. pallida*	QTL	spg	6	Caromel *et al.* 2003
GpaM3	*G. pallida*	QTL	spg	12	Caromel *et al.* 2003
R_{Mc1}	*M. chitwoodi*	M	blb	11	Brown *et al.* 1996 Rouppe van der Voort et al.1999
Fungus resistance					
Name	**fungus**	**type**	**source**	**chromosome**	**reference**
Sen1	*S. endobioticum*	M	tbr	11	Hehl *et al.* 1999

Oomycete resistance					
Name	**oomycete**	**type**	**source**	**chromosome**	**reference**
R1	*P. infestans*	M	dms	5	Leonards-Schippers *et al.* 1992
R2	*P. infestans*	M	dms	4	Li *et al.* 1998
R3a	*P. infestans*	M		11	Huang 2005
R3b	*P. infestans*	M		11	Huang 2005
R5	*P. infestans*	M		11	Huang 2005
R6	*P. infestans*	M	dms	11	El-Kharbotly *et al.* 1996
R7	*P. infestans*	M	dms	11	El-Kharbotly *et al.* 1996
R8	*P. infestans*	M		11	Huang 2005
R9	*P. infestans*	M		11	Huang 2005
R10	*P. infestans*	M		11	Huang 2005
R11	*P. infestans*	M		11	Huang 2005
	P. infestans	QTL	tbr/dms	8	Meyer *et al.* 1998
	P. infestans	QTL	tbr/spg	4/5	Leonards-Schippers *et al.* 1992
	P. infestans	QTL	tbr/spg	3/5/6/9/11	Oberhagemann *et al.* 1999
R12	*P. infestans*	M	brt	10	Sanchez *et al.* 2000
R13	*P. infestans*	M	brt	7	Sanchez *et al.* 2000
Rpi-ber1	*P. infestans*	M	ber	10	Ewing *et al.* 2000
RB	*P. infestans*	M	blb	8	Naess *et al.* 2000, Song *et al. 2003*
Rpi-blb1	*P. infestans*	M	blb	8	Van der Vossen *et al.* 2003
Rpi-blb2	*P. infestans*	M	blb	6	Van der Vossen *et al.* 2004
Rpi-blb3	*P. infestans*	M	blb	4	Park 2005
Rpi-abpt	*P. infestans*	M		4	Park 2005
	P. infestans	QTL	mcd	4/10	Sandbrink *et al.* 2000
	P. infestans	QTL	mcd		Bisognin *et al.* 2005
Rpi-moc1	*P. infestans*	M	moc	9	Smilde *et al. 2005*
Rpi-neo	*P. infestans*		neo	4/7	Smilde, unplublished results
Rpi-oka1	*P. infestans*	M	oka	9	Smilde, unplublished results
Rpi-oka2	*P. infestans*	M	oka	4	Smilde, unplublished results
	P. infestans	QTL	phu	7/12	Ghislain *et al.* 2001
Rpi1	P. infestans	M	pnt	7	Kuhl *et al.* 2001
R2-like	*P. infestans*	M		4	Park 2005

Virusresistance					
Name	**virus**	**type**	**source**	**chromosome**	**reference**
Ra_{adg}	PVA	M	adg	11	Hämäläinen *et al.* 1998
Rx1 (Rx)	PVX	M	adg CPC 1673*	12	Ritter *et al.* 1991, Bendahmane *et al.* 1997 Kanyuka *et al.* 1999
Rx2	PVX	M	acl MPI 44.1016/10	5	Ritter *et al.* 1991
Nb	PVX	M	tbr	5	de Jong *et al.* 1997
Nx_{phu}	PVX	M	phu, IVP35	9	Tommiska *et al.* 1998
Ns	PVS	M	DE 83-3121	8	Marczewski *et al.* 2002
R Y1	PVY	M			Vidal *et al.* 2002
Ry_{adg}	PVY	M	adg	11	Hämäläinen *et al.* 1997 Sorri *et al* 1999 Kasai *et al.* 2000
Ry_{sto}	PVY	M	sto	11	Brigneti *et al.* 1997
Ry_{chc}	PVY	M	chc	9	Hosaka *et al.* 2001
Ny_{tbr}	PVY	M	tbr	4	F. Celebi-Toprak *et al.* 2002
PLRV.1	PLRV	QTL	chc/yun	11	Marczewski *et al.* 2001
PLRV.4	PLRV	QTL	chc/yun	11	Marczewski *et al.* 2004

*pedigree not certain

The objective of this thesis is the development and implementation of molecular markers linked to the resistance against the root-knot nematodes *Meloidogyne hapla*, *M. fallax* and *M. chitwoodi*. The ultimate goal is the implementation of MAS in potato breeding in order to develop potato cultivars with resistance to these root-knot nematodes. The research was focussed on qualitative monogenic resistance as well as on quantitative resistance. In addition to the genetic analyses of the resistance, differential interaction with nematode populations was performed to predict the durability of the resistance. Chapter 2 describes the mapping of the quantitative resistance to *M. hapla* and *M. fallax* in a *S. chacoense* hybrid. The resistance genes detected as described in Chapter 2 are tested to different *M. hapla* populations in Chapter 3. Chapter 4 reports the mapping of another quantitative resistance against *M. hapla* in a *S. tarijense* hybrid. The mapping of the monogenic resistance against *M. chitwoodi* and *M. fallax* in *S. fendleri* is described in Chapter 5. In that report an interspecific and an intraspecific mapping population are used and results compared. Chapter 6 describes the mapping of another monogenic resistance gene to *M. chitwoodi* and *M. fallax* from *S. hougasii* and the variability of different *M. chitwoodi* populations to the resistance genes detected in *S. bulbocastanum*, *S. fendleri* and *S. hougasii*. Finally the implementation of markers linked to the detected resistance genes is discussed.

Chapter 2

Towards marker assisted selection for *Solanum chacoense* derived resistance against root-knot nematodes (*Meloidogyne* spp.) in cultivated potato.

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Abstract

The wild potato species *Solanum chacoense* contains resistance to the root-knot nematode species *Meloidogyne fallax* and *M. hapla.* To map the quantitative trait loci (QTLs) for this resistance, a genetic map of a segregating BC1 population was generated based on a total of 623 AFLP markers. Three QTLs were identified in this population. Two QTLs, $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}chc}B$, were explaining 38% and 13% of the total variance of the resistance to *M. hapla*. One QTL, $R_{Mf\text{-}chc}$, for resistance to *M. fallax* was identified, explaining 14% of the total variance. The map position of the QTL for resistance to *M. fallax* did not coincide with the positions of the QTLs for resistance to *M. hapla.* Due to the large differences in AFLP patterns between *S. chacoense* and *S. tuberosum* the identification of the linkage groups to potato chromosome nomenclature could not be established. Consequently chromosome numbers could not be assigned to the linkage groups containing the QTLs.

Introduction

The economic losses in potato cultivation due to nematodes are mainly caused by *Globodera spp.* and *Meloidogyne spp.* (Phillips 1994, Oerke *et al.* 1994). Resistance to nematodes is a major objective in potato breeding as a result of the reduced application of nematicides (Santo & O' Bannon 1981). Currently *Globodera* infestation is largely controlled by the combination of crop rotation and the cultivation of resistant potato varieties. Unfortunately *Meloidogyne* resistance is not available in cultivated potato and crop rotation does not offer a solution for *Meloidogyne spp.*, because of their polyfagous nature. In an earlier study a *Solanum chacoense* accession with a combined resistance against *M. hapla* and *M. fallax*, but susceptible to *M. chitwoodi* was identified (Janssen *et al.* 1997a). The same paper describes that the resistance to *M. fallax* in *Solanum* spp. was generally combined with resistance to *M. chitwoodi*. The localisation of the resistance gene against *M. fallax* on the same map position as the *M. chitwoodi* resistance gene, mapped by Brown *et al.* (1997) in *S. bulbocastanum* derived material, suggests that *M. chitwoodi* and *M. fallax* share virulence factors (Rouppe van der Voort *et al.* 1999). The absence of the generally combined resistance against *M. chitwoodi* and *M. fallax* in *S. chacoense* suggests that this resistance is effective to a different virulence spectrum. The introduction of this resistance is therefore desirable in potato breeding. However, introgression breeding requires time and labour consuming phenotypic screening for root-knot nematode resistance. In addition *M. fallax* is a quarantine organism, which puts certain restrictions to the bioassay. Application of molecular markers for the selection of resistant offspring genotypes offers the advantages of a rapid and non-laborious screening for resistance.

This paper describes the first step towards the development of marker assisted selection for the resistance. First, the inheritance of the *S. chacoense* derived resistance is studied. Second, the loci involved in resistance are mapped and finally the prospects of marker assisted breeding are discussed.

Materials & Methods

Plant material

The diploid (2n=2x=24) mapping population RH96-378 was obtained from a cross between the susceptible *S. tuberosum* female parent RH91-183-8 and the resistant male parent 87-206-6, an interspecific hybrid between *S. chacoense* accession BGRC18618 and the diploid *S. tuberosum* clone SH83-81-47. In previous experiments the hybrid 87-206-6 displayed a high level of resistance against *M. hapla* and was very vigorous (Janssen *et al.* 1996b). Approximately 1000 seeds were sown in vitro. The first germinating and most vigorous seedlings were multiplied in vitro to allow replicated resistance tests, genotype maintenance and tissue generation for DNA extraction. This resulted in a mapping population of 261 individuals.

Inoculum

Out of different race A types of *M. hapla*, the isolate Hi was chosen for the resistance test because of its high virulence (Van der Beek *et al.* 1998a). The *M. fallax* isolate Fb chosen for the resistance test was originally obtained from a soil sample in the Dutch town Baexem. Due to the lack of genetic variation within *M. fallax* (Van der Beek *et al.* 1997) the Fb population is assumed to be representative for all *M. fallax* populations in the Netherlands. The nematode populations were propagated on *Lycopersicum esculentum* cv. Motelle and were kindly provided by Dr. Hans van der Beek (Plant Research International, Wageningen).

Meloidogyne resistance test

The in vitro propagated cuttings were transplanted in clay pots of 350 cm^3 containing silver-sand and a slow release NPK fertiliser (Osmocote). During the first six days the relative humidity was kept close to 100%. The plants were grown in a greenhouse with a temperature ranging between 18 and 25°C. Four plants per genotype were tested for resistance against *M. hapla* and another four plants for resistance against *M. fallax* in a randomised block design. For the resistance test with *M. hapla*, there was insufficient plant material of the parents available. The resistant and susceptible phenotype of the parents was confirmed in a later experiment. In the resistance test with *M. fallax* both parents were included in 8 replications. In both resistance tests the susceptible variety Nicola was included to compare the number of egg masses with the amount of egg masses formed in the mapping population.

Four weeks after transplanting the in vitro plants into pots, the plants were inoculated with 1 ml of a water suspension containing approximately 500 second stage juveniles (J2s). Eight weeks later the root systems were rinsed with water to free them from sand and the egg masses were stained with phloxine-B (Dickson & Struble 1965) and counted. The numbers of egg masses was considered to be a measure of susceptibility and resistance. The size of the root systems was scored in 5 classes, ranging from 1: poorly developed to 5: very vigorously. Plants with a class 1 root system were discarded from further analysis.

DNA isolation and the AFLP protocol

DNA was isolated from frozen leaf material using a modified protocol of Van der Beek *et al.* (1992). The AFLP protocol was followed as described by Vos *et al*. (1995), except for the *EcoRI* adapter, which was used without the biotin label. The restriction enzymes *EcoRI* and *MseI* were used for the digestion of the genomic DNA. The *EcoRI* primers were radioactively labelled and the amplified fragments were visualised by autoradiography. The primer combinations used were E+AAA/M+ACG, E+AAC/M+CAC, E+AAC/M+CAG, E+AAC/M+CCA, E+AAC/M+CCT, E+AAG/M+CCT, E+ACA/M+CTA, E+ACC/M+AAG, E+AGA/M+CAT, and E+ATG/M+CTA. Six out of the ten primer combinations used in this study were also used to make a catalogue of AFLP markers covering the potato genome (Rouppe van der Voort *et al.* 1998a).

Data collection and linkage map construction

Autoradiograms with AFLP fingerprints were analysed visually with the help of the image analysis software Cross Checker (http//:www.dpw.wur.nl/pv/pub/CrossCheck/). This computer program simplifies the data collection and transforms the collected data in compatible data for Joinmap 2.0 (Stam & van Ooijen 1995), the computer program used to construct the genetic linkage maps. The genetic data were divided based on female and male gamete segregation in order to construct a paternal and maternal linkage map. Co-dominant AFLP markers segregating from both parents were scored dominantly and are therefore less informative as compared to the 1:1 markers. Those co-dominant markers, so-called bridge markers, segregating in a 3:1 ratio, were only added to the data set to align the two maps. For the analysis of marker distances those markers are omitted. After the determination of the marker order, the data were checked for the occurrence of singletons. All AFLP fragments giving rise to a singleton were inspected and if necessary rectified. The linkage groups were assigned to the potato chromosomes using locus specific AFLP markers from an online catalogue of AFLP markers (http//:www.dpw.wur.nl/pv/aflp/catalog.htm; Rouppe van der Voort *et al*. 1998a).

Statistical analysis

To normalise the data of the resistance tests, a square root transformation was performed on the number of egg masses counted per root system. Analysis of variance was carried out on the normalised data. The wide sense heritabilities (h^2) of the two resistance tests were calculated, with the formula $h^2 = \sigma^2(g) / [\sigma^2 (g) + \sigma^2 (e)]$.

The interval mapping method of MapQTL 4.0 (Van Ooijen & Maliepaard 2000) was applied to determine the LOD score and percentage of explained variance for the quantitative data of the nematode resistance. For the QTL analysis a selection of markers was made in such a way that a proportional distribution of markers over the linkage groups was obtained, with a mean interval length of approximately 5 cM. To establish an appropriate threshold value for declaring a significant QTL effect, the permutation test (Churchill & Doerge 1994) was performed as it is now available in MapQTL 4.0. The permutation test implies that the phenotypic data are shuffled, analysed for QTL effects at all markers in the set and the score saved. This procedure was repeated 1000 times. The 95% quantile was taken as an empirical obtained critical value at which the overall type I error rate for the experiment was 0.05 or less. If the LOD score exceeds this threshold, the presence of a QTL is inferred.

Results

Resistance tests

The number of egg masses observed in the progeny varied from zero to 107 and from zero to 143 for *M. hapla* and *M. fallax* respectively. In the resistant parent 87-206-6 the average number of egg masses over four replications was 0.5 egg masses of *M. hapla* and 0.0 egg masses of *M. fallax*. The mean number of egg masses formed on the susceptible clone RH91-183-8 was 37 and 64 for *M. hapla* and *M. fallax* respectively, whereas the susceptible potato variety Nicola showed 103 egg masses of *M. hapla* and 122 egg masses of *M. fallax*.

The average number of egg masses over the 4 replications differed significantly ($P<0.001$) between clones. The h^2 of the resistance against *M. hapla* and *M. fallax* was 53 % and 17 % respectively. No correlation between *M. fallax* and *M. hapla* resistance was observed. The frequency distribution of the mean square root of the counted egg masses of the progeny is shown in Figures 1 and 2. For both resistances the trait-values show a continuous distribution.

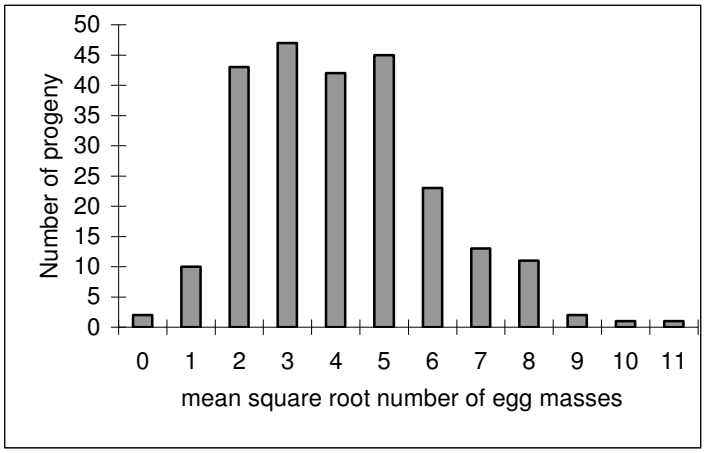

Figure 1: Frequency distribution of the mean square root of egg masses of M. hapla detected on the RH96-378 mapping population.

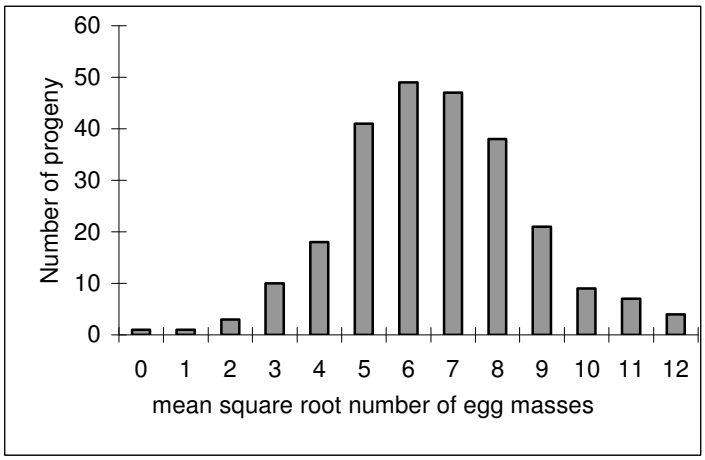

Figure 2: Frequency distribution of the mean square root of egg masses of M. fallax detected on the RH96-378 mapping population.

Linkage map construction

The use of 15 primer combinations revealed 313, 284 and 26 markers segregating from respectively the susceptible female parent, the resistant male parent and both parents. At a LOD score of 7 the maternal as well as the paternal markers group into 12 linkage groups with a total length of 685 cM and 596 cM respectively. In the map of the susceptible female parent 7 markers remained unassigned and in the map of the resistant male parent 9 markers remained unassigned. A high level of skewed segregation was observed, as only 44% of the markers fitted the 1:1 ratio ($P < 0.05$). The majority of the distorted segregations is from the hybrid parent (68%), in favour of the *S. chacoense* alleles. The similarity of the AFLP fingerprints between the resistant interspecific hybrid and the susceptible *S. tuberosum* clone was low. Approximately 4% of all polymorphic markers segregated from both parents. Usually the proportion of co-dominant markers in diploid potato crosses is ranging from 10% to 30%. Due to the low number of co-dominant markers, only five parental linkage groups could be aligned.

Chromosome alignment

Rouppe van der Voort *et al.* (1997a) state that when AFLP fragments have an equal electrophoretic mobility and target on the same genomic region, they are “common AFLP markers” and can be regarded as allelic. The alignment of the linkage groups to the potato chromosomes by this method was unsuccessful. Using the reference parents of the catalogue, only 51 of the 60 co-migrating markers of the female parent were common AFLP markers (Table 1). By means of those common AFLP markers 10 linkage groups of the susceptible parent could be assigned to a chromosome number. No linkage groups were assigned to chromosome 3 and 12. None of the 22 co-migrating markers of the interspecific hybrid resulted in an unequivocal identification of the linkage groups and consequently the chromosome numbers could not be assigned to the linkage groups using the catalogue. By means of the co-dominant markers five linkage groups of the interspecific hybrid are aligned with linkage groups of the susceptible parent. Those are assigned to chromosomes 4, 7 and 11, and the two unassigned linkage groups, chromosome 3 and 12.

Table 1: Number of co-migrating AFLP markers between the two parental clones and the 5 reference parents, with seven primer combinations. Some AFLP markers are present in more than one reference parent.

Parental genotypes	C	E	AM	RH	SH	Total
RH91-183-8	10	12	15	23	24	60
96-206-6	3	4	4	8	9	22

Localisation of QTLs involved in resistance

Because of the continuous variation of the amount of egg masses of *M. hapla* and *M. fallax*, it was impossible to make a clear segregation in resistant and susceptible progeny. Therefore the normalised data were used for quantitative trait mapping. For QTL analysis a selection of the markers was made in such a way that the mean interval length was approximately 5 cM to get a proportional distribution of markers over the linkage groups. The threshold value for declaring a significant QTL, calculated by the permutation test, was set on a LOD score of 4.4 and 4.2 overall for *M. hapla* and *M. fallax*. If the LOD score exceeds this threshold, the presence of a

QTL is inferred. The three linkage groups on which significant QTLs were detected are shown in Figure 3.

One QTL for resistance against *M. fallax* was identified, explaining 14% of the total variance. The two QTLs for the resistance to *M. hapla* were explaining 38% and 13% of the total variance. Two factor analyses of variance based on the amount of egg masses of *M. hapla* gave a significant interaction ($P<0.01$) between the two QTLs. In spite of its significance, the effect of this interaction was very small, so we may assume that the two genes generally act in an additive way. After the first map construction the QTL that explained 38% of the variance was mapped in an interval of 33.2 cM. In order to map the resistance gene more accurately, more AFLP markers were added to fill the gap. The primer combinations were selected via Bulked Segregant Analysis (Michelmore *et al.* 1991), with a bulk composed of 8 plants that amplified a band for both flanking markers and a bulk with 8 plants lacking the amplified band for both markers. Nine primer combinations out of 36 tested primer combinations, gave a bulk specific polymorphism, of which the 6 best scorable primer combinations were tested on the whole population. The addition of these markers to the linkage group lead to a smaller but still large interval of 17.1 cM. None of the 313 AFLP markers segregating from the susceptible maternal parent showed significant association with resistance.

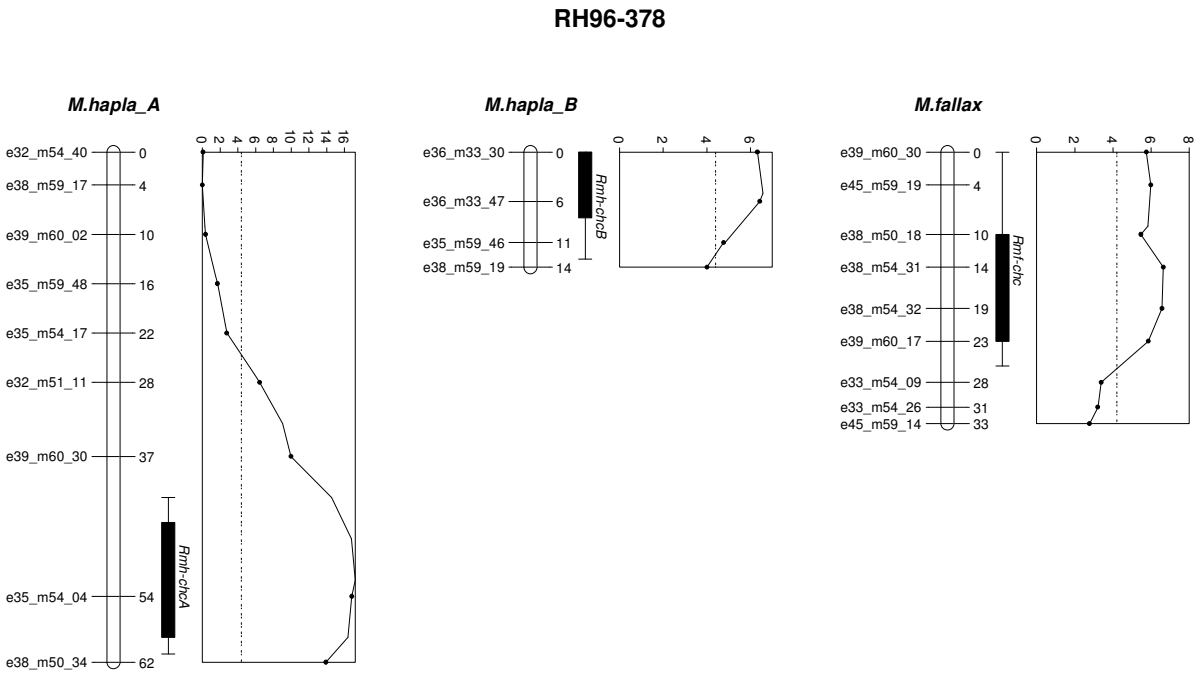

Figure 3: Three linkage groups of the RH96-378 mapping population with the positions of the QTLs $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}chc}B$, explaining respectively 38% and 13% of the phenotypic variance for resistance to *M. hapla*, and the QTL $R_{Mf\text{-}chc}$ explaining 14% of the phenotypic variance for resistance to *M. fallax*. Marker names are based on the primer combination and an arbitrary number. Distances between markers are given in centiMorgans. The QTLs detected by interval mapping are indicated with bars.

To detect minor QTLs the Multiple-QTL model (MQM) mapping method (Jansen 1994) of the software program MapQTL4.0 (Van Ooijen & Maliepaard 2000) was applied using the markers flanking the largest QTLs as cofactor. Only for the resistance to *M. hapla* a novel significant effect was detected that explained 9.6% of the variance. This QTL mapped on chromosome 7 and coincides with a QTL for root size. The positive correlation between the size of the roots and the number of egg masses was already noticed in previous statistical analyses (results not shown). We assume therefore that this QTL is involved in root-growth and consequently influences the amount of produced egg masses in a genotype.

The two QTLs conferring resistance to *M. hapla* allow a classification of offspring genotypes into a group having both QTLs, two groups with one QTL and a group without QTLs as shown in Figure 4. The average number of egg masses of the class with both QTLs, with the QTL having the largest effect, the QTL having the smaller effect and without a QTL are respectively 7.1, 11.6, 20.7 and 36.4 eggmasses. Within the classes there was a wide range of egg mass production. Genotypes of the four different classes also showed a big overlap in the mean number of egg masses produced.

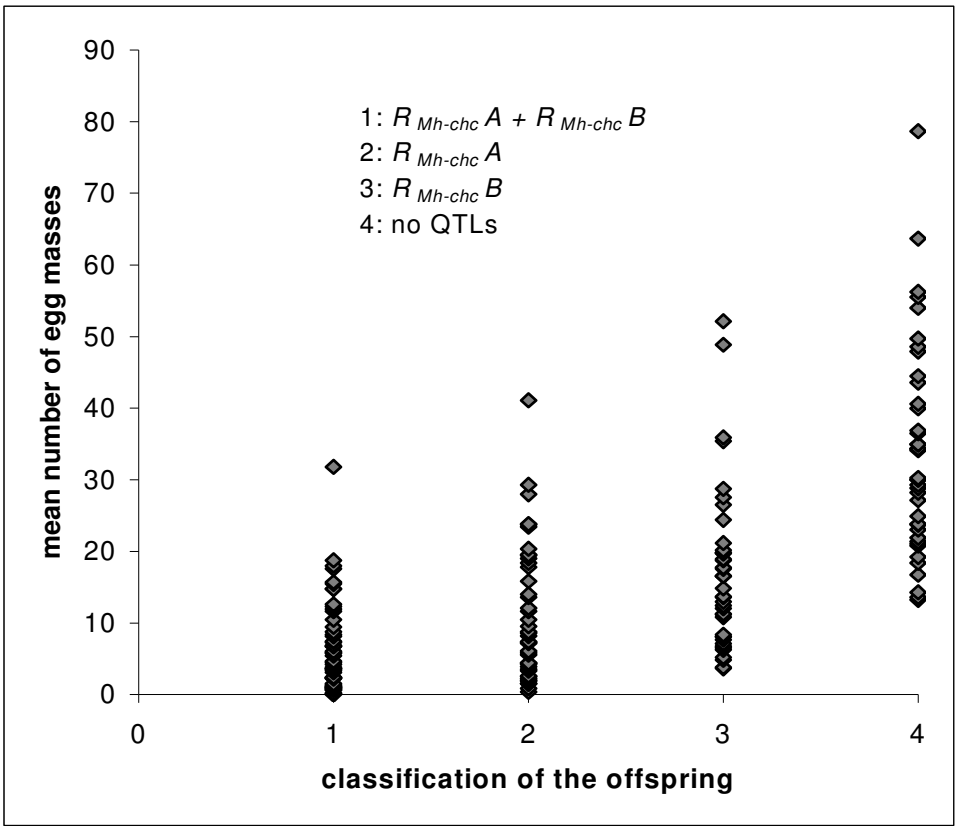

Figure 4: Mean number of egg masses per classification based on the genetic configuration of the offspring.

Discussion

The resistance to *M. hapla* of the clone 87-206-6, previously described by Janssen *et al.* (1996b), is confirmed in this research. The hybrid showed also resistance to *M. fallax*, comparable with the resistance of other *S. chacoense* accessions tested in that previous research. The relatively low number of egg masses in the susceptible parent can be explained by the small root system of genotype RH91-183-8. Root-size strongly affects the amount of produced egg masses on the plant and thus hampers the detection of true resistance genes.

In the mapping population a continuous distribution of the numbers of egg masses was found. Such a continuous distribution is expected when the resistance is controlled by QTLs. Another reason for the continuous distribution is the influence of environmental factors. High environmental variation and consequently low heritability of *M. fallax* resistance could cause to a large extent the continuous distribution of the number of egg masses found. Another factor causing a continuous distribution might be the heterozygosity of the *M. hapla* population. Van der Beek (1997) found within isolates of *M. hapla* a comparable large genetic variation as between isolates. When a *M. hapla* population is very heterozygous, it is plausible that it is also heterozygous for the avirulence genes. A mixture of nematodes with different avirulence genes can not only explain the continuous distribution but can also explain the low heritability. A continuous distribution of egg masses produced makes it impossible to form distinct groups of resistant and susceptible individuals and the results of such a resistance test should consequently be analysed with a QTL approach to localise the genetic factors of the resistance.

In order to perform the QTL analyses a genetic map was made with a total map length of 685 cM and 596 cM for respectively the female and the male map. The lengths of these maps are slightly smaller than other published genetic maps (Jacobs *et al.* 1995, Rouppe van der Voort *et al.* 1997b, Gebhardt *et al.* 1994), but we expect to have covered the potato genome sufficiently to allow the detection of all QTLs involved in these resistances. Seven out of 12 linkage groups of the resistant parent were to a large extent, or even completely, distorted. It was not unexpected that the linkage groups would have regions that show distorted segregation, because a large number of seeds did not germinate and approximately 20% of the seedlings did not form normal leaves after the cotyledon stage and were consequently discarded from further analysis.

The expectation was that approximately half of the polymorphic AFLP fragments of the interspecific hybrid 87-206-6 originated from *S. chacoense* and the other half from *S. tuberosum*. However over 80% of the polymorphic AFLP fragments was *S. chacoense* specific (results not shown). We don't have an explanation for this high amount of *S. chacoense* specific polymorphic AFLP fragments in the interspecific hybrid, by which as a consequence the AFLP fingerprints between the hybrid and the *S. tuberosum* clone largely differ. Due to this large difference in AFLP fingerprints "the comigrating AFLP marker approach" could not be used to assign chromosome numbers to the linkage groups. This was also the case in a mapping study with a *S. bulbocastanum* background (Rouppe van der Voort *et al* 1999). Clearly it is important that the *Solanum* species investigated is not too diverged from *S. tuberosum* to use "the co-migrating AFLP marker approach".

The results of QTL mapping show only one locus for *M. fallax* resistance, mapped on one of the distorted linkage groups. We propose to name this locus $R_{Mf\text{-}chc}$ in accordance to the proposed nomenclature for *R*-genes (Chapter 6). The linked markers showed a skewed segregation in favour of the resistant allele. In spite of the detection of the QTL in the majority of the genotypes of the mapping population, hardly any of these genotypes reached the resistance level of the resistant parent. Although the level of resistance is hardly maintained in the offspring, its value might still be useful for potato breeding. The *S. chacoense* resistance to *M. fallax* seems to differ in spectrum of resistance found in other *Solanum* spp.. In most cases resistance to *M. fallax* coincides with resistance to *M. chitwoodi*, however *S. chacoense* is susceptible to *M. chitwoodi* (Janssen *et al*. 1996a). The markers flanking the QTL for *M. fallax* resistance could be used for marker assisted selection (MAS). Taking into account the very low heritability that hampers the phenotypic selection and the assumed different resistance spectrum of *S. chacoense*, the introgression of $R_{Mf\text{-}chc}$ by means of MAS would be justified.

By means of QTL analyses two major QTLs were detected conferring resistance to *M. hapla*. The proportion of the phenotypic variation explained by these QTLs is comparable with the heritability, which is the proportion of the genotypic variance relative to the phenotypic variance. This suggests that the genome was sufficiently covered with markers and the offspring size was effective to identify all relevant loci involved in resistance. We name the resistance genes $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}chc}B$. According to the proposed nomenclature for R-genes (Chapter 6) suffixes could be added to the name, to indicate also the map position. Until a chromosome number is definitive assigned to the linkage groups we propose to add a letter to distinguish both QTLs. The two QTLs allow a classification of the offspring into four different groups having both $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}chc}B$, only $R_{Mh\text{-}chc}A$ or $R_{Mh\text{-}chc}B$ and a group without the two QTLs as shown in Figure 4. One plant seems phenotypically susceptible but is characterised as having both resistance genes. This genotype might be the result of double crossover event. With other nematode resistance genes it is known that even in shorter intervals such double crossover events seem to occur, although the susceptible phenotype might also be a result of genetic instability of the resistance alleles (Ballvora *et al.* 1995).

Figure 4 also demonstrates that phenotypic selection will lead to a frequent loss of one of the QTLs. Therefor the use of Marker Assisted Selection should be preferred, in order to decrease this risk. Based on a similarity study of Hospital & Charcosset (1997) the population size needed for introgression of resistances using molecular markers can be estimated. In that study the minimum population size required to obtain at least one individual with the resistance gene and all markers is calculated. A reasonable high value of this probability can be obtained in the first generation with no more than two markers per QTL. Implementing the results of the present study in the formulas presented by Hospital & Charcosset (1997) lead to high probabilities of having the desired genotype at the QTLs with a minimum population size of 21. A fast introduction of these QTLs by means of MAS should therefore be feasible, resulting in partial resistant commercial varieties.

Chapter 3

Differential interaction of the root-knot nematode *Meloidogyne hapla* with *Solanum chacoense* derived resistance genes

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Abstract

The differential interaction of eight *M. hapla* populations to the resistance QTLs, $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}chc}B$ from the wild potato species *S. chacoense* and the molecular variation of the *M. hapla* populations were studied. The $R_{Mh\text{-}chc}A$ allele allows recognition on virulence factors present in the six tested cytological race A populations. Only in the population initially used for the QTL analysis, an additive effect of the minor QTL $R_{Mh\text{-}chc}B$ was found. No effect of both alleles was noticed for the two race B populations. Knowledge of these virulence factors in the nematode populations and the complementary resistance genes are tools for identification and resistance breeding. Based on the molecular variation of the *M. hapla* populations the cytological races A were divided in a different group than the populations of race B. The cytological races A and B can thus be divided based on their differences in virulence to the $R_{Mh\text{-}chc}A$ allele and based on molecular differences. The use of a pathotype scheme and the gene pool similarity concept are discussed.

Introduction

Root-knot nematodes *Meloidogyne* spp. are globally distributed pathogens, of which *M. hapla* is most commonly found in areas of potato cultivation and can cause severe yield reduction (Brodie 1984, MacGuidwin & Rousse 1990). These losses can be restricted by crop rotation; however the wide host range of *M. hapla* limits a practical application of this strategy. The use of nematicides seems a more consistent way of controlling the root-knot nematode, but is environmentally unfriendly and should be avoided as much as possible. The most desirable and effective method of nematode control is the use of resistance. Unfortunately no acceptable level of resistance is found in commercial potato cultivars. Recently, in the wild potato species *Solanum chacoense* two QTLs were detected for resistance to the *M. hapla* population Hi (Chapter 2).

The durability of this resistance is depending on the genetic diversity for genes involved in virulence of the nematode and the selection for virulent genotypes upon exposure of specific resistance genes. In potato isolate-by-cultivar interaction was detected, suggesting the existence of variation in virulence genes in *M. hapla* (Van der Beek *et al.* 1998a). Also several factors involved in (a)virulence were detected in a study with wild *Solanum* species (Van der Beek *et al.* 1998b). The two races of *M. hapla*: race A which is facultative meiotic parthenogenetic and race B that is obligate mitotic parthenogenetic, show both a similar range of (a)virulence genes (Van der Beek *et al.* 1998b). Assuming that a number of different (a)virulence genes exist in *M. hapla*, it is important to arrive at a reliable pathotype scheme that can help potato breeders in pyramiding resistance genes in cultivars to cover a broad spectrum of virulence factors. For that purpose the nematode populations should be assigned to pathotypes based on a combination of molecular data and virulence characteristics. Similar studies to reveal the relationship between genetic diversity across nematode populations and

virulence factors have been conducted in potato cyst nematodes (Blok & Phillips 1995; Folkertsma *et al*. 1996b; Thiery *et al.* 1997). In a similarity study based on the two-dimensional gel electrophoresis method little intraspecific variation was observed within different *M. hapla* populations but a clear separation could be made between race A and race B (Van der Beek *et al.* 1997).

In this paper we describe experiments to analyse the interaction between *M. hapla* and two resistance QTLs from the wild potato species *S. chacoense* as revealed in a previous study (Chapter 2). The variation in virulence will be compared with the molecular variation present in diverse *M. hapla* populations. An estimation of the genetic diversity of the investigated *M. hapla* populations will be made based on an AFLP fingerprint.

Materials & Methods

Potato genotypes

Two QTLs, $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}chc}B$, conferring resistance to the *M. hapla* population Hi have been identified in the mapping population RH96-378 (Chapter 2). RH96-378 is a cross between the susceptible diploid *S. tuberosum* genotype RH91-183-8 (2n=2x=24) and the resistant parent 87-206-6, an interspecific hybrid between *S. chacoense* accession BGRC18618 and the diploid *S. tuberosum* clone SH83-81-47.

Based on the AFLP-data, the individuals of the BC1 population could be divided in four different groups: a group having both QTL-alleles: $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}chc}B$, a group with the $R_{Mh\text{-}chc}A$ allele, a group with the $R_{Mh\text{-}chc}B$ allele and a group without the two alleles associated with resistance at both loci. Initially six genotypes from each group were selected and multiplied in vitro. One genotype from the group having both alleles hardly grew in vitro and was discarded from further examination. One genotype with allele $R_{Mh\text{-}chc}B$ was incorrectly assigned as having no resistance. Another genotype from the group with both QTLs was tested again with AFLP and turned out to lack $R_{Mh\text{-}chc}A$. In the end 4, 6, 8 and 5 genotypes of the group containing both alleles, the $R_{Mh\text{-}chc}A$ allele, the $R_{Mh\text{-}chc}B$ allele and group without the two alleles respectively, were transplanted in clay pots of 350 cm^3 containing silver-sand and a slow release NPK fertiliser (Osmocote). During the first six days in a greenhouse, the relative humidity was kept near 100%. Five plants per genotype were tested for resistance against different *M. hapla* populations in a randomised block design. The two parents of the genotypes and the susceptible potato cultivar Nicola were included. The transplantation of the in vitro plants into the silver-sand resulted in the death of several plants. Due to the unsuccessful transplantation together with the plants with a poor root development 15% of the plants were discarded from analysis. The remaining plants were analysed statistically.

Nematodes

Eight *M. hapla* populations have been used in this study (Table 1). They were collected from different hosts and different geographical sites in the Netherlands and from several countries around the world. The 8 populations Ham, Han, Hao, Haw, Hb, Hbs, Hbz and Hi, used in the virulence test were maintained and propagated on *Lycopersicum esculentum* cv. Moneymaker.

Table 1: The origin and races of the *M. hapla* populations. The cytological race A and B were distinguished by studying the meiosis (Van der Beek 1997).

Population	origin	Last host	race
Ham	Australia	unknown	A
Han	South Korea	Hosta	B
Hao	Heide, the Netherlands	unknown	A
Haw	France	Grape	A
Hb	Zwaanshoek, the Netherlands	Astilbe	A
Hbs	Horst, the Netherlands	Fennel	A
Hbz	Amerzoden, the Netherlands	Rose	B
Hi	Smilde, the Netherlands	Tomato cv. Motelle	A

Virulence test

Four weeks after transplanting the in vitro plants, an aliquot of approximately 500 second-stage juveniles (J2s) were inoculated into small holes around the stem of the plants. Eight weeks later the roots of the plants were rinsed free from sand and stained with Phloxine-B (Dickson & Struble, 1965). Individuals with a very small root system were omitted for further examination. The numbers of egg masses were counted as a measure of virulence/resistance.

DNA isolation & AFLP analysis

DNA isolation of the nematodes in J2 stage was performed according to Zijlstra *et al.* (1995) and checked on agarose gel to determine the DNA concentration. Samples with no visible DNA were precipitated with an equal amount of isoamylalcohol and pelleted in a microcentrifuge. The pellet was dissolved in 10 µl deionised water and directly used for AFLP template preparation. From the other samples approximately 50 ng genomic DNA was used for the AFLP template preparation. The AFLP procedure was performed following the protocol as described by Vos *et al*. (1995). The restriction enzymes used were *EcoRI* and *MseI*. The pre-amplification was performed using the primers E+0/M+C, followed by PCR amplification using the M+CAC primer and a ^{33}P-labeled E+A primer. A water control was included in the AFLP analysis. The AFLP autoradiogram was evaluated using the image analysis software Cross Checker (http//:www.dpw.wur.nl/pv/pub/CrossCheck/). The AFLP markers were dominantly scored for each nematode population as 1 (band present) or 0 (band absent) for similarity analysis. A neighbour joining tree was calculated using the computer program TREECON (Van de Peer & De Wachter 1994).

Results

The virulence of the nematodes and the resistance of the potato genotypes was analysed by counting the egg masses on the root system. The average numbers of egg masses are listed in Table 2. The results of individual plants tested in previous research with the Hi population are included for the sake of comparison, because Hi was the population used to select the QTL in the first place (Chapter 2). The susceptible potato cultivar Nicola was used as susceptible control in this experiment, although there is no guarantee that this genotype is void of R-genes against all populations of *M. hapla.* Similarly the susceptible diploid clone RH91-183, previously used as a parent to develop the population for the detection of the QTLs involved in resistance against *M. hapla*, was added. Nicola and RH91-183 proved to be the most susceptible genotypes for the nematode populations Han, Haw, Hb and Hbs. In the case of population Hao and Haw the multiplication on susceptible controls does not exceed the susceptible experimental materials, but the number of egg masses is still in the same range.

A significant effect of the $R_{Mh\text{-}chc}A$ allele could be found in the populations Hi, Ham, Hao, Haw, Hb and Hbs. The $R_{Mh\text{-}chc}B$ allele has a significant effect on the populations Ham and Hi, but no significant effect could be found in population Ham between the three different resistant classes. Only in population Hi the additive effect of the $R_{Mh\text{-}chc}B$ allele was found. No significant effects of the two alleles on the production of egg masses of the two race B populations Han and Hbz could be found. Although the experimental design does not allow statistical analyses of differences in aggressiveness, large variation is detected in the ability to form egg masses on the tested potato genotypes.

Table 2: The average of egg masses of eight different *M. hapla* populations of race A or B on the genotypes with both resistance alleles, with one resistance allele and without the resistance alleles, and on one resistant (R) and two susceptible (S) controls. For each *M. hapla* population separately, means carrying a different letter, are significantly different from each other (P<0.05).

	race A	race A	race A	race A	race A	race A	race B	race B
	Hi	Hb	Hbs	Haw	Hao	Ham	Han	Hbz
$R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}chc}B$	1.7 a	2.3a	2.7 a	3.2 a	5.0 a	0.2 a	28.0 a	5.5 a
$R_{Mh\text{-}chc}A$	4.8 b	2.1 a	0.9 a	8.0 a	6.4 a	0.7 a	33.5 a	1.5 a
$R_{Mh\text{-}chc}B$	14.9 c	31.1 b	13.4 b	31.0 b	24.0 b	1.0 a	32.7 a	2.4 a
No resistance	44.3 d	28.28 b	9.7 b	20.6 b	15.0 b	5.1 b	34.7 a	3.6 a
87-206-6 (R)	0.5	0.0	0.2	0.0	0.8	0.0	18.4	0.6
RH91-183 (S)	37.3	77.4	6.0	37.4	10.7	4.3	50.6	0.0
Nicola (S)	103.3	143.6	148.8	23.5	20.8	0.6	37.4	7.0

Molecular diversity across the nematode populations was analysed with molecular markers. The followed DNA extraction method was very laborious and not always successful. Unfortunately the DNA of the nematode population Hi could not be isolated. Of the remaining 7 nematode populations one AFLP fingerprint was generated with one primer combination. Because the bands in the upper and in the lower part of the AFLP autoradiogram were not clear in all 7 lanes, only fragments between 100 and 330 basepairs in the middle section of the AFLP fingerprint were analysed. In this section the analysis of the AFLP data revealed 127 clear AFLP fragments of which 99 were polymorphic across the investigated populations. A dendrogram was constructed following the neighbour joining tree method Figure 1. The two nematode populations Han and Hbz are remarkably different as compared to the other populations. The *M. hapla* race A populations are clustered together.

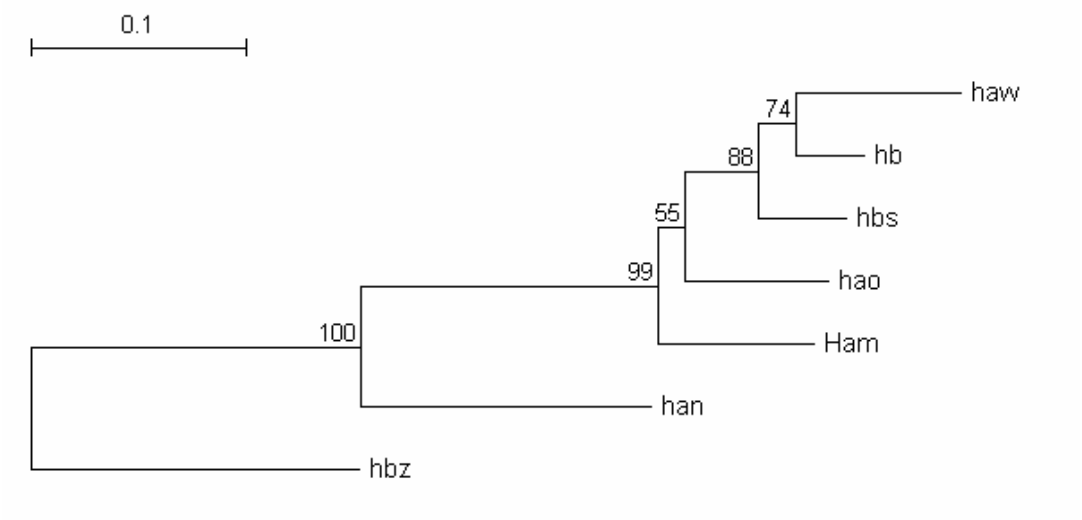

Figure 1: Neighbour joining tree of the seven *M. hapla* populations based on DNA fragments from AFLP analysis. Percentages of the bootstrap values are mentioned above the branches.

Discussion

It seems that the $R_{Mh\text{-}chc}A$ allele allows recognition on virulence factors present in the race A populations. No effect of both alleles was noticed for the race B populations. Also the resistant parent was susceptible for the race B population Han, which makes it unnecessary to screen the *S. chacoense* population with such a race B population. The two races used to be distinguished on their mode of reproduction. With the $R_{Mh\text{-}chc}A$ allele we can now also discriminate between the two races based on their reproduction. The correlation between sexual reproduction and virulence is intriguing, but so far without explanation.

The $R_{Mh\text{-}chc}B$ allele of the minor QTL has an insignificant effect on the *M. hapla* populations tested. Only in population Hi and Ham there is an effect of the $R_{Mh\text{-}chc}B$ allele causing a reduction of the egg masses produced. It is obvious that this allele should have an effect on population Hi because this was the initial population used to identify and map the QTLs. There is no indication that additional QTLs were present in the *S. chacoense* mapping population. Although the $R_{Mh\text{-}chc}B$ allele has an effect on population Ham, this allele has no significant additional effect in combination with the $R_{Mh\text{-}chc}A$ allele.

The differences in egg mass production, irrespective of the genetic composition of the individual plant, indicate the variation in aggressiveness between the populations. Indeed large differences in aggressiveness exist between the tested nematode populations. This has also been reported by Van der Beek *et al*. (1998a) where a race B nematode population showed a much smaller reproduction factor as compared with the other *M. hapla* populations tested. From a breeders point of view those populations are of little concern. It is unlikely that the growing of resistant varieties would change the population in such a way that it will become more aggressive.

Knowledge of the virulence factors in nematode populations and the complementary resistance genes would provide tools for identification and pyramiding the genes in a variety. Therefore a reliable pathotype scheme would be valuable to the breeder. Kort *et al*. (1977) proposed a pathotype scheme for *Globodera* spp. based on the quotient between the final and initial population density (Pf/Pi values) on a set of differentials, discriminating five pathotypes of *G. rostochiensis* and three of *G. pallida*. This pathotype scheme has been criticized because of the arbitrary use of the Pf/Pi values which is environmentally sensitive and because of the use of differential clones with quantitatively based polygenic resistance (Trudgill 1985). *G. pallida* populations with a similar pathotype, based on the scheme of Kort *et al.* (1977), are often molecular very distinct (Folkertsma *et al.* 1994) and they frequently have a different response to various sources of resistance.

An alternative to the pathotypes scheme is the gene pool similarity concept (Bakker *et al*. 1993) using molecular techniques to establish the similarity between nematode populations. In absence of selection pressure the molecular similarity of the populations will be a reflection of the virulence loci. This concept was confirmed testing the genetic similarities of 102 Dutch

potato cyst nematode populations and their virulence towards four resistant potato cultivars (Folkertsma 1997). The potato cyst nematodes differ however in dispersal and reproduction with the root-knot nematodes. Considering the broad host range of the root-knot nematode, these nematodes will multiply almost every year and will have more generations each year, whereas the potato cyst nematode will reproduce only during the growth of potatoes and only one generation a year. The dispersal of root-knot nematodes is also higher compared with potato cyst nematodes because they can be dispersed by the tubers, whereas the potato cyst nematode only disperses with the tuber in the attached soil. Due to the higher reproduction and greater dispersal the effects of mutation and selection on a population will be higher compared with the potato cyst nematode. However we expect that the existing virulence characteristics in these populations, before the introduction of resistant cultivars, are the major force behind the "resistance-breaking" nematode populations. Analyses of the virulence related polymorphisms between the nematode populations will therefore predict if new resistance genes have added value.

Although we have limited genetic dataset, large differences between the *M. hapla* populations have been found. The largest differences have been observed between the two cytological races A and B. We could confirm the results of Van der Beek *et al.* (1997) and divide the nematode populations of race A in a different group than the race B populations. The cytological races A and B can thus be divided based on their differences in virulence to the $R_{Mh\text{-}chc}A$ allele and based on molecular differences.

One of the aims of this research was to establish the relationships between the diverse populations based on AFLP data in order to use that information in testing the effectiveness of new sources of resistance. Our results show that the breeder has to use race B populations in order to detect new sources of resistance. According to our results the population Han should be preferred because of its combination of virulence and aggressiveness.

Chapter 4

Genetic analysis of the *Solanum tarijense* derived resistance to the Northern Root-Knot Nematode *Meloidogyne hapla* in a potato BC1 population.

Jan Draaistra, Ronald C.B. Hutten, Joost Riksen, Paul H.G. van Koert, Herman J. van Eck

Abstract

A non absolute level of resistance to the Northern root-knot nematode *Meloidogyne hapla* was observed in the wild *Solanum* species *S. tarijense*. In order to perform marker assisted selection for the introduction of this resistance into the susceptible cultivated potato (*S. tuberosum*), a diploid BC1 mapping population was developed and analysed for its resistance to *M. hapla*. The mapping population showed a continuous distribution of the number of egg masses. A genetic map was constructed using AFLP markers and the resistance was mapped in a quantitative as well as in a qualitative method. The QTL mapping approach resulted in one locus and this was confirmed later with the qualitative mapping approach. The genetic map was successfully aligned to the genetic map of a catalogue population. The linkage group with the mapped resistance gene could therefore be placed on the distal position of the short arm of chromosome 7. The resistance gene is named $R_{Mh\text{-}tar}$ and is the first described resistance gene mapped on the short arm of chromosome 7.

Introduction

The Northern root-knot nematode *Meloidogyne hapla* is a common pest in potato producing areas with a temperate climate. It is able to reproduce on a broad range of dicotyledous hosts and can lead to severe economic losses. Potato is a very good host of *M. hapla* and the nematode can multiply extensively during the growth of potato. Under natural cropping circumstances *M. hapla* is able to reproduce up to three generations in one growing season of potato. The major damage in potato caused by the nematode is the reduction of yield which can increase up to 70% reduction in a highly infested plot (MacGuidwin & Rouse 1990, Stirling & Wachtel 1985). Furthermore the high number of nematodes that remain in the soil after growing a potato crop will cause severe problems when the next year susceptible crops like carrots or chicory are grown.

In order to control the nematode population the use of nematicides is very effective but for environmental reasons less desirable. Alternative control strategies are crop rotation with monocotyledons and the use of resistant varieties. Minor differences in susceptibility to *M. hapla* have been observed in commonly grown potato varieties (Van der Beek *et al.* 1998a), but these differences are too small to be able to select useful levels of resistance. In several wild *Solanum* species a non absolute level of resistance to *M. hapla* has been observed, among others in *S. tarijense* (Janssen *et al.* 1996a).

Currently, breeding for resistance to *M. hapla* in potato is relying exclusively on bioassays. A disadvantage of the bioassay is the use of natural nematode populations that are probably a mixture of undetermined races of the nematode. Moreover the bioassay in the greenhouse is expensive and laborious. A far better alternative for the bioassay is the application of molecular marker assays to discriminate between resistant and susceptible plants.

In this paper we describe the genetic analysis of resistance in a diploid population derived from a backcross between *S. tuberosum* and a F1 hybrid of the wild species *S. tarijense*. The non absolute level of resistance combined with a continuous distribution of produced egg masses in

the segregating offspring (unpublished results), suggests that the resistance is under polygenic control. Therefore QTL analysis is pursued using a linkage map well covered with AFLP markers, based on a high number of offspring genotypes. The detection of QTLs is the first step in a marker assisted selection approach, to be used for the introgression of *M. hapla* resistance in potato.

Materials & Methods

Plant material

The diploid mapping population RHAM061 was obtained from a cross between the resistant parent RH90-011-4 and the susceptible *S. tuberosum* genotype RH89-039-16. The resistant interspecific hybrid RH90-011-4 is obtained from a cross between *S. tarijense* (BGRC 24717) and a dihaploid clone of the cultivar Mondial. Seeds of the mapping population were sown in vitro and the seedlings were multiplied in vitro in order to obtain sufficient plantlets for the resistance test and genotype maintenance as well as to generate tissue for DNA extraction.

Resistance test

Three weeks old in vitro cuttings were transplanted in clay pots of 350 cm^3 containing silver-sand and a slow release NPK fertiliser (Osmocote), and grown with a relative humidity near 100% during the first six days. The temperature in the greenhouse was maintained at 20°C, with fluctuations of 4°C. Together with the two parents and 228 offspring, three susceptible potato varieties were included in the resistance test: Bintje, Agata and Donald. Most genotypes were tested in four replications in a completely randomised design. Plants showing inferior growth were omitted from further analysis. Four weeks after transplanting, the plants were inoculated with 1 ml of a water suspension containing approximately 400 J2 nematodes of *M. hapla* population Hb. Population Hb is a race A of *M. hapla* that was originally collected from a soil sample near the Dutch town Zwaanshoek (Van der Beek *et al.* 1998a). The nematode population was propagated on *Lycopersicum esculentum* cv. Motelle and kindly provided by Dr. Hans van der Beek (Plant Research International, Wageningen). Eight weeks after inoculation the root systems were rinsed and stained with Phloxine-B (Dickson & Struble 1965). The amount of egg masses was determined as a measure of susceptibility. To investigate the data with an analysis of variance, the number of egg masses counted per root system was first normalised with a square root transformation. From the ANOVA the wide sense heritability (h^2) of the resistance test was estimated.

Map construction

The AFLP protocol was followed as described by Vos *et al*. (1995), using the restriction enzymes *PstI* and *MseI* to digest the genomic DNA. The *PstI* primers were radioactively labelled and the amplified fragments were visualised by autoradiography. All primer combinations were analysed before on the SH×RH population (Rouppe van der Voort *et al.*

1997a, 1998a, 1998b; Isodore *et al.* 2003; http//:www.dpw.wur.nl/pv/aflp/catalog.htm), with RH being the abbreviated name of RH89-039-16. Therefore most of the chromosomal positions of the AFLP markers are already known which enables the assignment of a chromosome number to the different linkage groups. The primer combinations used were P+AC/M+ATC, P+AG/M+AAG, P+AG/M+AAT, P+AG/M+ACC, P+AG/M+AGA, P+AG/M+AGC, P+AG/M+AGG, P+AG/M+AGT, P+CA/M+AGT and P+TG/M+ACT. The analyses of the autoradiograms were done visually with the help of the image analysis software Cross Checker (http//:www.dpw.wur.nl/pv/pub/CrossCheck/). The mobilities of the AFLP markers were estimated, using the mobility of markers mapped in the SH×RH population. The two parental genetic maps were made using the computer program Joinmap 2.0 (Stam & van Ooijen 1995). Therefore the genetic data were divided based on female and male gamete segregation. In order to align the paternal and maternal linkage map co-dominant AFLP markers segregating from both parents were used as bridge markers, segregating in a 3:1 ratio. For the final calculation of map distances the bridge markers are omitted because of their less informative nature.

QTL mapping

The data of the resistance test were analysed with the interval mapping method of MapQTL 4.0 (Van Ooijen & Maliepaard 2000) for QTL detection. The interval mapping method of MapQTL 4.0 determines the LOD scores and percentages of explained variance for the quantitative data. To obtain an even distribution of markers over the linkage groups, a linkage map was constructed with a mean interval length of approximately 5 cM. Using the permutation test (Churchill & Doerge 1994), with a 95% quantile as an empirical obtained critical value at which the overall type I error rate for the experiment was 0.05 or less, a critical LOD value was set. When a LOD profile exceeded this threshold, the presence of a QTL is inferred. In addition to the interval mapping method, the multiple QTL mapping (MQM) module of MapQTL 4.0 was applied. The marker nearest to the largest QTL was taken as a cofactor to eliminate the major part of the variation induced by that QTL and subsequently enhance the detection of minor QTLs on other linkage groups.

In an attempt to locate the resistance locus more precisely, the phenotypic data were analysed qualitatively. A subset of the complete data set was used for this analysis. Only the offspring genotypes of which the data of 3 or 4 replications are known and having a fair root system were used to minimise the chance of false identification of resistant genotypes. The large group of individuals that had a mean number of egg masses between 1 and 10 were also discarded for the qualitative analysis. The trait value below 1 egg mass were considered resistant and values higher than 10 egg masses as susceptible.

Results

Phenotypic data

The mean number of egg masses produced on the resistant and susceptible parent was 0.5 and 32 respectively. The mapping population showed a continuous distribution of the number of egg masses. The mean number of egg masses counted on the offspring genotypes varied between 0 and 69.25. The most susceptible F1-plant exceeded the number of egg masses formed on the susceptible controls Agata, Bintje and Donald, having a mean of respectively 52, 40 and 33. Analysis of variance of the square root normalised data showed a significant ($P<0.01$) genetic variance in the mapping population. The heritability of the resistance was estimated to be 0.40.

Genetic linkage mapping

A genetic linkage map was constructed using Joinmap 2.0 (Stam & van Ooijen 1995) on the basis of 265 segregating markers on the population of 182 individuals. When the LOD score exceeded 3.0, linkage between AFLP markers was inferred. With this LOD threshold 82 markers (aa×ab) were placed on the RH89-039-16 map, 134 markers (ab×aa) were placed on the RH90-011-4 map and 9 markers remained unassigned. A skewed segregation ratio (χ^2 test, $P<0.001$) was observed in 14%, 20% and 25% of the respectively ab×aa, aa×ab and ab×ab markers. The majority of the markers that show a skewed segregation ratio are linked and mapped on chromosome 6 and 8 of the resistant parent and on chromosome 3, 4 and 12 of the susceptible parent. On both parental maps the markers were evenly distributed, showing hardly any clustering. Because many AFLP markers on the RH89-039-16 map have known map positions, the assignment of a chromosome number to the linkage groups was simple. Subsequently, the identity and orientation of the RH90-011-4 linkage groups could be determined by aligning the two parental maps with 39 out of the 40 ab×ab markers by using these markers as allelic bridges. After the alignment, these markers were discarded for further analysis.

QTL analysis

The interval mapping method showed significant association between markers and resistance on one of the twelve chromosomes of the resistant parent only. The region with this significant effect was located on chromosome 7, explaining 19.7 % of the variation. To determine whether the genetic effects were solely controlled by this region, MQM was performed. The marker P13M41_05 closely linked to the QTL on chromosome 7 was used as a cofactor. No other locus was detected that was significantly involved in resistance using the MQM method.

Qualitative mapping

Besides by means of QTL analysis, resistance was also mapped in a qualitative way. To avoid misclassifications, only the extreme trait values of the resistance test were converted into

qualitative data. The amount of less than1 egg mass for resistant and more than 10 egg masses for susceptibility are used as arbitrary thresholds. The subset of the population contained 88 individuals which is large enough to use for mapping analysis. The resistance is segregating in a Mendelian way 1:1 (χ^2 = 1.64). The resistance analysed with the qualitative mapping approach revealed a linkage group with 12 AFLP markers (Figure 1). In Figure 1 the three bridge markers used to align the two parental linkage groups are included. Three markers, indicated in italics, of the susceptible parent are present in the Ultra High Density (UHD) map of SH×RH (http://www.dpw.wur.nl/uhd/). These markers are placed in so-called Bins. The AFLP marker P+AG/M+AGT_83.8 is mapped in the centromere (Bin 68) of the UHD map. Based on the marker information of the UHD map, the resistance locus can be placed on the distal position of the short arm of chromosome 7. The markers P+AG/M+AAG_32 and P+AG/M+AGG_05 are closely linked to the resistance. Recombinants between the two markers and the resistance gene are present, however no feasible solution in marker order could be found. Therefore the resistance gene and both markers are presented as one locus. Further research is needed to determine the correct ordering of these loci.

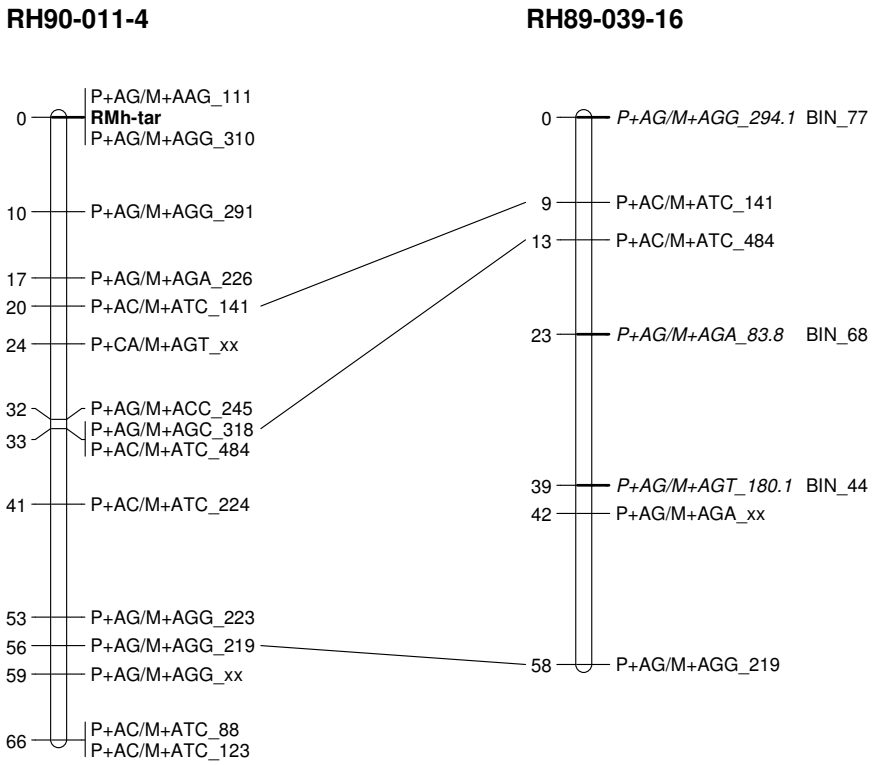

Figure 1: The genetic map of chromosome 7 of the resistant parent RH90-011-4, including the resistance locus R_{Mh_tar}, and the susceptible parent RH89-039-16. The AFLP markers mapped in the SH×RH population are indicated in italics. The Bin numbers that contain these markers are mentioned behind the marker names. Chromosome orientation is according to Dong *et al.* (2000). AFLP-markers of which the sizes are not estimated are marked by xx.

Discussion

The estimated broad sense heritability of 0.40 indicates that the amount of egg masses is highly variable and probably strongly influenced by the environment. Based on the low heritability one might conclude that the resistance test was not reproducible. Such low heritabilities have been found in previous resistance tests used to map *M. hapla* resistance in another population (Chapter 2). There are many environmental factors influencing the outcome of a resistance test, although the complete randomisation of the test should minimise these effects. Clearly these low heritabilities show that the use of a bioassay for estimating resistance to *M. hapla* is not suitable in a potato breeding program. Therefore the results underline the necessity of linked molecular markers in order to apply marker assisted selection in the breeding program.

The high variability of the resistance test is also a drawback in the mapping of the resistance. Moreover the results show that on the resistant parent some egg masses are formed as was already noticed by Janssen *et al* (1996a). In their research on average 1.4 egg masses were observed on the resistant parent, where 0.5 egg masses were observed in this study. The successful propagation of a limited number of nematodes on the resistant parent can be explained when assuming the presence of a low frequency of virulent nematodes in the initial population. The nematode population used in this experiment was obtained from a soil sample and could be a mixture of nematode genotypes with different (a)virulence genes. However the current lack of resistance in potato makes it difficult to specify this variation in virulence.

In order to localise the gene(s) involved in resistance a genetic map was constructed. This map was used both for MapQTL analysis as for Joinmap analysis. The diploid potato clone RH89-039-16 was used as susceptible parent because of its existing AFLP map, which is an advantage in map construction. The AFLP markers could be divided easily among the 12 linkage groups based on the previous mapping studies of RH89-039-16 (Isodore *et al.* 2003; http//:www.dpw.wur.nl/pv/aflp/catalog.htm). The locus specific AFLP markers made it easy to assign the correct chromosome numbers to the linkage groups. The resistant parent map could be aligned straightforward by using the ab×ab markers as allelic bridges. This mapping study resulted in two genetic maps of 786 cM and 606 cM for respectively the resistant and the susceptible parent. These map lengths are, although slightly shorter, comparable with the map lengths presented in other studies (Rouppe van der Voort *et al.* 1997a, 1998a; Celis-Gamboa *et al.* 2002). The results show distorted markers in both parents, which is a common feature in potato (Jacobs *et al.* 1995, Bonierbale *et al.* 1994). By choosing the *PstI* restriction enzyme as a rare cutter for template preparation, we observed hardly any centromeric clustering of AFLP markers as compared with *EcoRI* based markers (Van Eck *et al.* 1995, Rouppe van der Voort *et al.* 1997a). This decreased clustering of markers was also noticed in other crops like maize (Vuylsteke *et al*. 1999).

With respect to the covering of AFLP markers over the twelve chromosomes, we assume to have a suitable genetic map to perform MapQTL. The genetic map combined with the phenotypic data should allow the detection of all loci involved in resistance. However the results of the QTL analysis did not indicate that the resistance to *M. hapla* was controlled in a polygenic way. It was surprising that only one interval with significant association with the resistance was detected. The finding of this QTL did not explain all the genetic variation present in the mapping

population. However also with the multiple QTL mapping (MQM) method (Jansen 1994), which greatly enhances the power of detection of minor QTLs, no other QTLs contributing to the resistance could be detected. This does not imply that other QTLs are absent in this mapping population. Minor QTLs may stay unnoticed, especially in view of the large amount of residual variance caused by the environmental influence on the resistance. Most likely the value of other QTLs, if present in this population, will be of minor importance for breeding purposes considering the impossibility of mapping them.

The detection of just one QTL explaining the resistance, justifies the attempt of mapping this resistance gene by treating the resistance as a qualitative trait. The arbitrary threshold of 1 egg mass represents 0.25% successful reproduction of the total number of inoculated juveniles, which is comparable with other research. Janssen *et al.* (1996a) used a threshold of 1% reproduction of the total number of inoculated juveniles to distinguish between resistance and susceptibility. Using the subset of 88 individuals the resistance gene could be mapped more precisely. In this research the marker data of the UHD map could be successfully used in order to determine the chromosome number and the orientation of the linkage group. The resistance locus is on the distal position of the short arm of chromosome 7. The orientation of the linkage group as presented in Figure 1 is inverted relative to most of the literature references where TG20 is north and TG61 is south. However, the north and south arm of chromosome 7 as presented in Figure 1 correspond cytogenetically with the long and short arm respectively, according to Dong *et al.* (2000). Previously only a few other resistance genes have been mapped to this linkage group: the potato cyst nematode resistance *Gro1* (Barone *et al.* 1990) and *S. berthaultii* (Sanchez *et al.* 2000), *S. pinnatisectum* (Kuhl *et al.* 2001) and *S. phureja* (Ghislain *et al.* 2001) derived late blight resistance. According to the proposed nomenclature for *R* genes (Chapter 6), we will name the resistance gene $R_{Mh\text{-}tar}$. In order to use a marker assisted selection (MAS) approach for the introgression of this resistance gene in potato more closely linked markers are needed.

Considering the low heritability of the resistance test, a resistance gene like this should be incorporated in potato with MAS instead of the phenotypic bioassay. The practical value of the $R_{Mh\text{-}tar}$ resistance gene for potato cultivation will depend on the allele frequency of a matching virulence within the *M. hapla* populations and the distribution of such nematode populations. In order to develop resistant potato varieties with a broad resistance spectrum against different *M. hapla* populations, it is recommended to combine the $R_{Mh\text{-}tar}$ resistance gene with other resistance genes from other *Solanum* species. This is however only profitable when the resistance genes are complementary to the matching virulence in the nematode population. The detection of closer linked markers and subsequently designing closely linked CAPS markers will open the possibility to combine these different resistance genes in potato. Besides producing an assumable higher yield, resistant varieties can establish a reduction of residual population density that subsequently will cause less damage to the next crop in rotation. Further research should be carried out to assess differential interaction and synergic effects to a wide range of different *M. hapla* populations.

Chapter 5

Analysis of inter-specific and intra-specific crosses to localise the *Solanum fendleri* derived resistance gene $R_{Mc1\text{-}fen}$ against *M. chitwoodi* and *M. fallax* on potato chromosome *11*

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Abstract

An inter-specific and an intra-specific mapping population of the tetraploid species *S. fendleri* were developed for the genetic localisation of the gene $R_{Mc1\text{-}fen}$, conferring resistance to *Meloidogyne chitwoodi* and *M. fallax*. Results of the bio assay allowed a discrete classification of resistant and susceptible phenotypes. The resistance to both nematode species was in both mapping populations absolutely correlated. With Bulked Segregant Analysis a linkage group could be formed with AFLP markers comprising the $R_{Mc1\text{-}fen}$ locus. The use of a simple single locus specific PCR marker assay derived from the conversion of a relatively large AFLP fragment, in a reference population made it possible to align the linkage group to chromosome 11. The resistance gene mapped on a distal position of the long arm of chromosome 11, a region that is known to contain many resistance genes. The newly developed primer pair, however, could not distinguish the resistant and susceptible alleles in the mapping population. Further sequence analysis is necessary to regain this discrimination and to develop primers to use in marker assisted selection. The differences between the intra-specific and inter-specific mapping populations are discussed.

Introduction

Nematode species are important world-wide pests of potato. Various options for controlling and reducing their damage include the use of nematicides, deep ploughing, crop rotation and resistant cultivars. The growing of resistant cultivars is the most desirable and effective method of control. Due to substantial breeding efforts a large number of cultivars are currently available with adequate levels of resistance against the potato cyst nematode species *Globodera rostochiensis* Stone and *G. pallida* Woll.. The cultivation of these potato cyst nematode resistant cultivars allowed a decrease of the application of nematicides, but resulted in an increase of other nematode species, including the root-knot nematodes *Meloidogyne chitwoodi* Golden and *M. fallax* Karssen. These nematodes are of growing concern on the sandy and sandy-peat soils in the Netherlands (Molendijk & Mulder 1996). In the pacific North-West of the United States the root-knot nematode *M. chitwoodi* is already an important pest (Evans & Trudgill 1992). Recently it has been discovered in Texas as well (Szalanski *et al.* 2001). The broad host range of the *Meloidogyne* spp. impedes the control by means of crop rotation. Therefore the development of resistant cultivars is highly desirable. Unfortunately, no resistance to *M. chitwoodi* and *M. fallax* has been found in commercial potato cultivars (Janssen *et al.* 1995), but screening several wild *Solanum* species revealed resistance to these nematodes (Janssen *et al.* 1996a; Brown *et al.* 1988, 1991). One of these resistance genes, the R_{mc1} locus, originating from *S. bulbocastanum* (2n=2x=24), has been mapped on chromosome 11 (Brown *et al.* 1996; Rouppe van der Voort *et al*. 1999). In the wild species *S. fendleri* (2n=4x=48) the gene R_{mc2} was found, which provides resistance to both *M. chitwoodi* and *M. fallax* (Janssen *et al*. 1997b).

The rapid introgression of resistance genes and efficient selection of resistant offspring in commercial breeding programs would greatly benefit from indirect selection with molecular markers. Bulked Segregant Analysis (BSA)(Michelmore *et al*. 1991) allows a targeted

identification of molecular markers with close linkage to the trait locus while minimising experimental work. However, BSA was originally designed for disomic inheritance. BSA will loose much efficiency when applied to tetraploid organisms, because simplex/nulliplex (*Aaaa × aaaa*) markers are required, with the A-allele being linked in coupling phase with the simplex target allele. Li *et al.* (1998) deduced (assuming that 25 % of the bands in AFLP fingerprints show a polymorphism), that the expected proportion of simplex/nulliplex markers is approximately 5 %. Only a quarter of the simplex/nulliplex markers will have the correct linkage phase.

Two different strategies can be pursued to generate a mapping population for the localisation of a wild species derived resistance gene. The first option is directed towards the identification of a resistant and a susceptible genotype within or between wild species gene-bank accessions. Next, these genotypes are crossed and the intra-specific offspring is evaluated to test for segregation of the target gene. Depending on the zygosity of the target locus and the ploidy level of the species, further backcrosses may be required to find a convenient segregation ratio. The level of polymorphism could be lower in the intra-specific mapping population, because *S. fendleri* is a self-pollinator, so an elevated level of homozygosity is expected.

The alternative option is introgression of the gene into the cultivated species by recurrent backcrosses, while screening for the presence of resistance. A commonly observed drawback of inter-specific introgression material is the reduced level of recombination between the introgression segment and the recipient genome (De Vincente & Tanksley 1991; Ganal & Tanksley 1996). Inter-specific backcrosses can be complex because of crossing barriers, and it will take several generations. However, this is not a waste of time, because introgression is also a goal on its own. Moreover, it will automatically change the original zygosity of the target gene and markers into the simplex/nulliplex condition required for BSA.

In this paper we compare both the intra-specific and the inter-specific approach and evaluate both systems on their merits. The level of polymorphism is compared between the intra- and inter-specific mapping population. In addition we will evaluate the level of recombination in the intra- and inter-specific mapping population, and utilize high levels of recombination to obtain the resolution required for map based cloning.

Materials & Methods

Plant material

Two different mapping populations were developed for the genetic localisation of the root-knot nematode resistance gene of *S. fendleri* (Janssen *et al*. 1997b; Brown *et al.* 1999). The design and parental genotypes of the inter-specific and intra-specific mapping populations are presented in Figure 1. The inter-specific BC_2 population RH4X-088 and the intra-specific BC_1 population M95-236 consisted of 121 and 83 offspring genotypes respectively.

The tetraploid *S. fendleri* genotypes 93-114-5 and 93-114-11 are the donors of resistance in the mapping populations. They originated as seedlings from the *S. fendleri* gene-bank accession BGRC8083 and were previously tested by Janssen *et al.* (1997b). On the basis of previous

research (unpublished results), the resistant genotypes M94-51-1 and RH4X-036-11 were expected to be simplex (Rrrr). Consequently both mapping populations are expected to segregate in a 1:1 ratio.

The seeds of the mapping population RH4X-088 were sown in vitro, and cuttings were multiplied on MS30 medium. Three weeks old in vitro cuttings were transplanted in the greenhouse in 350 cm³ clay pots containing silver sand and slow release NPK fertilizer ('Osmocote', Sierra Chemical Company, Milpitas, USA). During the first six days the relative humidity was kept near 100%. The temperature in the greenhouse was maintained at 20° C, with fluctuations of 4°C. The seeds of mapping population M95-236 were sown in soil, and cuttings were made for the resistance tests. Further conditions were similar to the RH4X-088 population. The mapping populations were tested in a completely randomised design with 3 to 4 replicates of each clone.

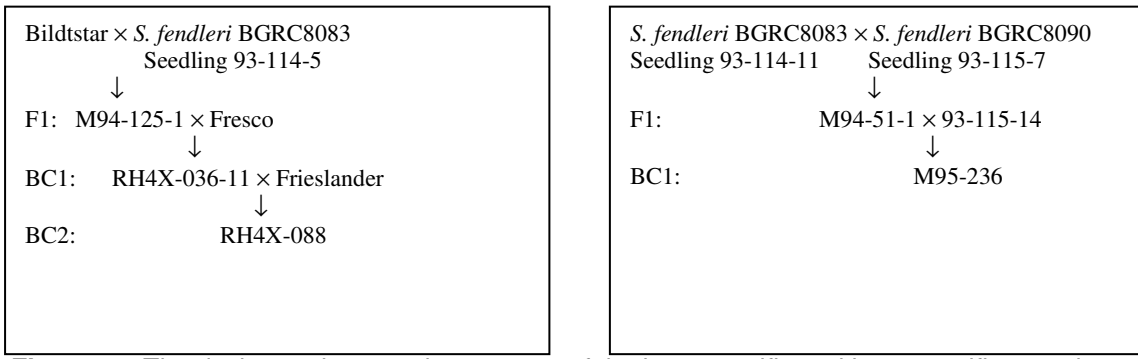

Figure 1: The design and parental genotypes of the inter-specific and intra-specific mapping populations

Meloidogyne resistance test

Four weeks after transplanting, the plants were inoculated with 1 ml of a water suspension containing approximately 500 juvenile (J2) larvae. All plants were tested for resistance against *M. chitwoodi* population "Co" (formally known as "CHE", Janssen *et al.* 1998) and *M. fallax* population "Fa" (formally known as "CHB", Janssen *et al.* 1998). Eight weeks after inoculation the root systems were rinsed and stained with Phloxine-B (Dickson & Struble 1965). The number of egg masses on resistant and susceptible clones is ranging from 0-11 and 25-250, respectively. This allows a discrete classification of resistant and susceptible phenotypes on the basis of the number of egg masses. Plants with a poorly developed root system were omitted from further analysis.

Marker analysis

Total genomic DNA was extracted from leaf material as described by van der Beek *et al.* (1992). The AFLP technique was performed according to Vos *et al.* (1995). The AFLP template was prepared using the enzyme combinations *Eco*RI/*Mse*I and *Pst*I/*Mse*I. Nomenclature of the AFLP markers is based on the template enzyme combination abbreviated to one letter, followed

by letters that represent the selective nucleotides and a number describing the visually estimated mobility of the fragment relative to the bands of the SequaMark 10 base ladder (Research Genetics, Huntsville, AL, USA). BSA was performed by pooling equal aliquots of secondary template of eight random chosen resistant and eight random chosen susceptible plants of the BC_2 population RH4X-088. For the intra-specific population M95-236, the bulks consisted of 10 plants. Usually the two parents are included during a BSA experiment to allow detection of markers in coupling as well as in repulsion phase. At the tetraploid level, linked markers in repulsion will not show bulk specificity and therefore inclusion of parental samples in BSA was irrelevant. The inter-specific mapping population RH4X-088 was tested with 44 primer combinations. The intra-specific population M95-236 however, was screened with 304 of the 512 possible primer combinations of *Eco*+ANN and *Mse*+$^{A}/_{C}$NN. AFLP based on *Pst*I/*Mse*I template was applied on the RH4X-088 population only. In this second round of screening, two susceptible and one resistant bulk were screened with all 256 combinations of 16 M+CNN primers combined with 16 P+NN primers. AFLP primer combinations with a bulk specific amplification product, indicating a putatively linked marker, were verified on the individual genotypes of the bulks. Only those primer combinations without a recombinant in these individuals were applied on the complete mapping population. The chromosome number of the linkage group was confirmed by testing the chromosome 11 specific CAPS marker M39b (Brigneti *et al*. 1997) using the PCR conditions as described by the authors. The M39b PCR products were digested with the restriction enzyme *DdeI* and separated on a 1.5 % agarose gel. The genetic data of the marker loci and phenotypic resistance data were analysed using Joinmap version 2.0 (Stam & van Ooijen 1995).

Conversion of the AFLP marker into a simple single locus specific PCR marker assay.

For the conversion of AFLP markers into a simple marker assay, AFLP bands were excised from dried polyacrylamide gels and diluted in 100 µl of TE for 1 h at room temperature. The excised AFLP fragments were re-amplified using elongated AFLP primers. These M13 AFLP primers have an extra stretch of nucleotides at the 3' end, complementary to the M13 primer site, to allow the sequencing of the PCR products with standard M13 sequencing primers. Each band was sequenced at least twice. When possible, a primer pair was developed with an annealing temperature of at least 45°C. Primers with a lower annealing temperature are more sensitive to mismatch and will consequently produce additional non-specific products.

The ultra dense map of potato (Isidore *et al.* 2003; http//:www.dpw.wur.nl/pv/aflp/catalog.htm) was used as a reference population to enable the alignment of the linkage group to the correct potato chromosomes.

Results

Inheritance of resistance to M. chitwoodi and M. fallax

Most genotypes of the mapping population, that were evaluated for resistance to *M. chitwoodi* and *M. fallax,* could be clearly classified as resistant or susceptible (Table 1). Occasionally some egg masses were formed on resistant genotypes but never more than 11, whereas in the susceptible genotypes over 200 egg masses could be found. The Chi-square test showed that the segregation of resistant and susceptible phenotypes did not deviated from a 1:1 ratio in the intra-specific population M95-236 (χ^2=1.39; p=0.24). A significant deviation from a 1:1 segregation ratio (χ^2=5.83; p=0.016) was observed in population RH4X-088 where an excess of susceptible genotypes occured. The segregation of the resistance to both nematode species *M. chitwoodi* and *M. fallax* was absolutely correlated.

Table1: Number of resistant, susceptible and unknown genotypes of the inter-specific mapping population RH4X-088 and intra-specific mapping population M95-236.

	Resistant	Susceptible	Unknown
RH4X-088	45	71	3
M95-236	41	31	11

AFLP analysis

Resistant and susceptible bulks of the intra-specific population M95-236 were screened with 304 *Eco/Mse* AFLP primer combinations, and only one AFLP marker with linkage to the resistance gene was obtained. The 44 *Eco/Mse* primer combinations that were tested in the inter-specific population RH4X-088 resulted in eight bulk specific amplification products. When tested on the complete offspring, four AFLP bands appeared to be false positives. The remaining four AFLP markers showed significant linkage with the locus involved in resistance and had a similarly skewed segregation pattern. The second round of BSA of population RH4X-088 with 256 *Pst/Mse* primer combinations resulted in 15 additional bulk specific bands. Seven of these primer combinations showed AFLP markers without recombination in the subset of 16 genotypes of the bulks. These seven primer combinations were tested on the complete offspring and tight linkage was confirmed between these seven markers and the resistance locus.

The marker E+ACT/M+AAC_85 was the only marker that was obtained in the intra-specific population. The marker mapped at a distance of 2.5 cM from the resistance locus. In the inter-specific map this marker was not observed using BSA, because another AFLP-fragment from one of the susceptible *S. tuberosum* parents with almost the same mobility was segregating. The presence of this *S. tuberosum* derived AFLP band overshadowed the presence or absence of the *S. fendleri* derived AFLP band that was linked to the resistance locus. Marker E+ACT/M+AAC_85 was scored afterwards in the inter-specific population, in those individuals that did not have the *S. tuberosum* derived AFLP fragment. The estimated genetic distance between the resistance locus and this marker was 2 cM. None of the ten AFLP markers linked with the resistance locus in the inter-specific population was polymorphic in the intra-specific population.

Genomic localisation of the resistance locus

To identify the chromosome number of the linkage group, three AFLP fragments were excised from gel and sequenced to allow the development of locus specific PCR primers. Only one primer pair, developed on the sequence of the AFLP marker P+GG/M+CAC_302, amplified a marker that was polymorphic in the reference mapping population SH×RH (Isidore *et al.* 2003). The PCR products gave polymorphic bands after digestion with restriction enzyme *MboI*, resulting in two bands of approximately 200 and 150 bp (Figure 2). Parent SH had both bands ($A_{200}A_{150}$) while parent RH only had the lower band (A_{150}). To explain the segregation of 76 offspring genotypes, the following model was most plausible.

$A_{200}A_{150} \times A_{150}A_{null}$ = ¼ $A_{200}A_{150}$ + ¼ $A_{200}A_{null}$ + ¼ $A_{150}A_{null}$ + ¼ $A_{150}A_{150}$, of which the latter two can not be distinguished.

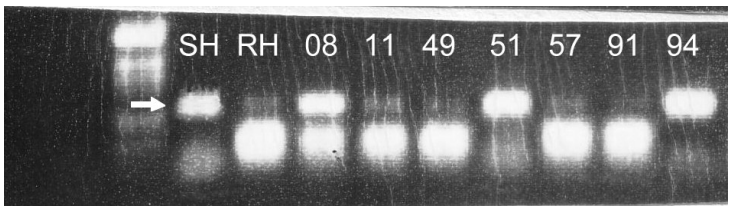

Figure 2: Part of the 1.5 % agarose gel with the PCR product with different segregation types in the mapping population SH×RH, obtained with the primer pair based on the sequence of AFLP marker P+GG/M+CAC_302, after digestion with restriction enzyme *MboI*. The segregating PCR product is indicated with an arrow.

The expected ratio was 1:1:2 and the observed ratio 22:23:31 fitted this model (χ^2=2.61, p=0.27). The A_{200} allele could be mapped in bin SH11_05 at a distal position on the long arm of chromosome 11. Therefor, in reverse the chromosome number of the linkage group of population RH4X-088 comprising the resistance locus was confirmed by testing the chromosome 11-specific CAPS marker M39b (Figure 3, Brigneti *et al*. 1997).

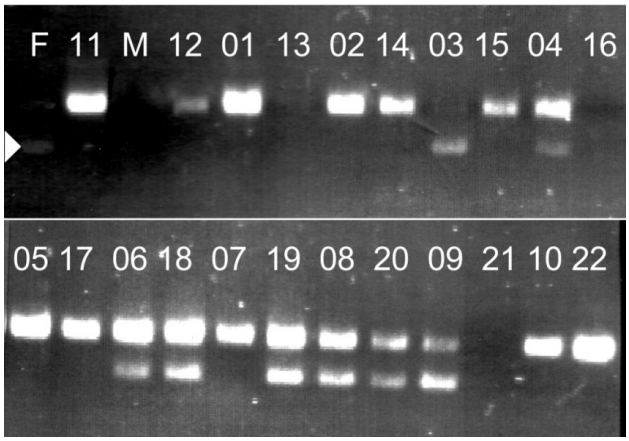

Figure 3: Part of the 1.5 % agarose gel with the PCR product segregating in the inter-specific mapping population, obtained with the M39b primerpair. The mapped PCR product is indicated with a triangle.

In the progeny no recombinants were detected between the M39b marker and the resistance locus. The construction of a linkage group of the inter-specific population, comprising eleven AFLP markers, M39b and the resistance locus resulted in a map with a total length of 11 cM (Figure 4). According to the proposed nomenclature for *R* genes (Chapter 6), we will name the resistance gene $R_{Mc1\text{-}fen}$.

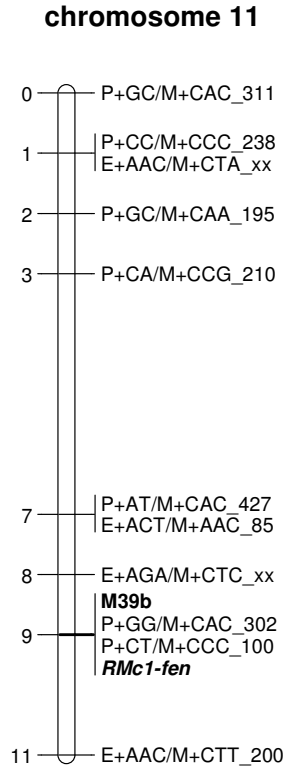

Figure 4: Position of the $R_{Mc1\text{-}fen}$ locus on the long arm of chromosome 11 of the resistant clone RH4X-088. Chromosome orientation is according to Dong *et al.* (2000). AFLP markers of which the sizes are not estimated are marked by xx.

Amplification products derived from a PCR with the primers based on the sequence of AFLP marker P+GG/M+CAC_302, were also segregating in the mapping population (Figure 5). This primer pair gives one band of approximately 100 bp in the resistant parent of the inter-specific population and one band with the size of approximately 200 bp in the susceptible parent. In the mapping population the marker segregates in four phenotypic classes with: both bands, one band of the resistant parent, one band of the susceptible parent or without amplification of a band. In combination with the phenotypic data the progeny could be divided in six classes (Table 2).

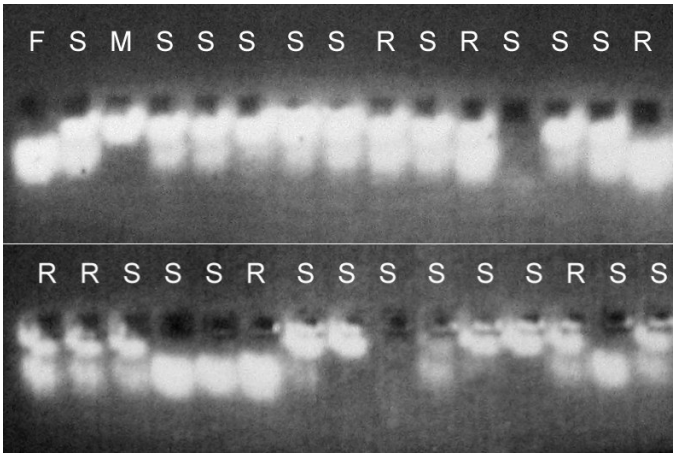

Figure 5: Parts of the 1.5 % agarose gel with the PCR product with different segregation types in mapping population RH4X-088, obtained with the primer pair based on the sequence of AFLP marker P+GG/M+CAC_302. The resistant female parent RH4X-036-11 is indicated with a F, the susceptible male parent Frieslander is indicated with a M. The resistant and susceptible progenies are indicated with respectively a R and a S.

Table 2: number of progeny per distinguished classe with the primer pair based on the sequence of AFLP marker P+GG/M+CAC_302, and the phenotypic data.

Phenotypic data	Amplification product	number of progeny
Resistant	100 bp, 200 bp	24
Resistant	100 bp	14
Susceptible	100 bp, 200 bp	32
Susceptible	200 bp	8
Susceptible	100 bp	11
Susceptible	No band amplified	2

Discussion

In this study, two different strategies have been compared to localise a gene involved in resistance to the root-knot nematode species *M. fallax* and *M. chitwoodi*. One striking difference was the level of polymorphism between the two populations. Only one AFLP marker was detected in the intra-specific mapping population. The low level of within-species polymorphisms, as reflected by the low number of bulk-specific AFLP markers, is clearly the result of the reproduction mode of *S. fendleri*. As a rule of thumb self-fertilising species show a lower level of genetic polymorphisms. On the other hand the genetic dissimilarity between the wild species introgression segments and the recurrent potato parents may have caused an elevated level of polymorphic markers in the BC_2 inter-specific mapping population.

Evaluation of the assumed reduction of the level of recombination in inter-specific hybrids is somewhat complicated, because the intra-specific map is composed of only one marker and the $R_{Mc1\text{-}fen}$ locus. The intra-specific map distance of 2.5 cM is slightly larger than the distance of 2 cM in the inter-specific map. Although in addition three markers of the inter-specific map cluster without recombination with the R-gene, we feel that the amount of data is inadequate to conclude that the results render to support for reduction in the level of recombination.

Most relevant from the breeder's point of view is the following. The use of inter-specific mapping populations allows the parallel development of a mapping population, with the development of resistant breeding lines. In addition, markers that show close linkage in experimental material can be used in breeder's material without validation. Finally, the marker data allowed the selection of genotypes RH4X-088-50 and RH4X-088-87 using marker P+AT/M+CAC_427 which has a recombination event adjacent to the $R_{Mc1\text{-}fen}$ locus, thus minimising wild species linkage drag.

Our data confirm the monogenic resistance to the two nematode species, as suggested by Janssen *et al.* (1997b). In the same paper Janssen *et al.* showed that the resistance to *M. fallax* and *M. chitwoodi* is correlated, as is confirmed by our results in both mapping populations. This correlation could mean that the resistance gene is involved in recognition of a similar avirulence gene product from both *M. chitwoodi* and *M. fallax*. It is not unlikely for the two nematode species, being very close related, to have similar avirulence gene products. Rossi *et al.* (1998) showed that the nematode resistance gene *Mi* of tomato also confers resistance to the very distinct related potato aphid. Apparently the avirulence gene products of these two organisms, which belong to different phyla, are very similar. On the other hand, it is not unlikely that avirulence genes of *M. chitwoodi* and *M. fallax* differ. Besides isozyme band pattern, the two species are also distinguished by their differential host ranges, which might be due to a different set of avirulence genes. It is shown that maize is a non-host for *M. fallax* whereas it is a good host for *M. chitwoodi* (Van Meggelen *et al.* 1994). In the case that *M. chitwoodi* and *M. fallax* really differ in their set of avirulence genes, the correlated resistance to both nematode species could be based on different *R*-genes that are members of the same resistance gene cluster. The cloning of the $R_{Mc1\text{-}fen}$ resistance gene in conjunction with a complementation assay will provide the opportunity to find out if the same gene regulates resistance to both nematode species, or that two different genes are present in the same cluster.

Occasionally some egg masses were formed on resistant genotypes of either mapping population. In a greenhouse experiment, the selection of virulence in *M. chitwoodi* towards the $R_{Mc1\text{-}fen}$ gene was easily achieved (Janssen *et al.* 1998). The nematode population used in our study is a field population with an unknown composition of virulent and avirulent genotypes. Considering the unequivocal difference between resistant and susceptible plants, the fraction of resistance breaking nematodes is not high. Most likely one growing season with a $R_{Mc1\text{-}fen}$ resistant potato cultivar will impose a strong selection, leading to an increasing proportion of virulent genotypes. However the current absence of any resistance in potato necessitates the application of the $R_{Mc1\text{-}fen}$ gene, although the selection of virulence by resistant cultivars should be monitored.

We successfully converted an AFLP marker into a single locus PCR marker. With this converted primer pair three alleles could be distinguished by a band of 200 bp, a band of 100 bp and no band. The genetic model of the resistant parent of the inter-specific population is L^RL^VII, where L^R is the resistant allele showing a band of 100 bp and L^V is the susceptible allele showing the same 100 bp band. Because the resistant and susceptible alleles L^R and L^V cannot be distinguished, this marker can not be used for marker assisted selection. Further sequence analysis is necessary to regain the discrimination between the susceptible and resistant alleles. Therefore we will sequence the flanking DNA of the AFLP marker and subsequently construct a SNP based on the discriminating basepairs of the resistant and susceptible genotypes. Because we did not detect a recombination event between the converted AFLP marker and the resistance gene, the SNP developed on the flanking DNA will be useful for marker assisted introgression of the resistance in potato.

The markers detected with BSA represented alleles from *S. fendleri* origin and did not coincide with AFLPs that have been observed in earlier studies. Therefore it was not possible to align the linkage group containing the $R_{Mc1\text{-}fen}$ locus with existing AFLP linkage maps. If co-migrating alleles from *S. tuberosum* origin would have been observed, from which the map position was known, this would have provided an indication for the chromosomal position of this linkage group (Rouppe van der Voort *et al.* 1997a). Previous research showed the failure of using this strategy to align *S. bulbocastanum* alleles to the *S. tuberosum* linkage map. This was probably due to the distant relation of the two species (Rouppe van der Voort *et al.* 1999). Although *S. fendleri*, in contrast to *S. bulbocastanum*, is cross compatible with *S. tuberosum*, the genetic distance to *S. tuberosum* is apparently also too large to alllign linkage maps by means of common AFLP markers.

Based on the sequence of a relative large AFLP fragment (302 bp), we were able to develop a primer pair, that gave in a diploid potato population (SH×RH) rise to segregating PCR products. With segregation analysis the linkage group could be aligned to chromosome 11, which could be confirmed by the chromosome 11 specific CAPS marker M39b. According to Dong *et al.* (2000) the physical orientation of chromosome 11 has an opposite orientation to the current potato genetic linkage group. The RFLP marker GP125 is localised on the long arm of chromosome 11, but in current linkage groups on the north arm. The marker M39b is linked at the distal side to GP125. The resistance gene $R_{Mc1\text{-}fen}$ should therefore be mapped on a distal position on the long south arm of chromosome 11. This is a region that is known to contain many resistance genes. In the same region the $R_{Mc1\text{-}blb}$ gene is mapped, a resistance gene of *S. bulbocastanum* which gives also resistance to *M. chitwoodi* and *M. fallax* (Brown *et al.* 1996; Rouppe van der Voort *et al.* 1999). It is common feature that resistance genes are clustered in potato. Resistance genes like *R3*, *R6* and *R7*, which give resistance to different races of *Phytophthora infestans*, are located next to each other in a narrow genome segment (El-Kharbotly *et al*. 1994, 1996). The found locus $R_{Mc1\text{-}fen}$ coincides with a resistance gene cluster containing genes of related and non-related pathogens. Besides the resistance gene $R_{Mc1\text{-}blb}$, the potato cyst nematode resistance gene *Gpa3* (Wolters *et al*. 1998), the wart disease resistance gene *Sen1* (Hehl *et al*. 1999), the virus resistance genes Ry_{adg} (Hämäläinen *et al*. 1997), Ra_{adg} (Hämäläinen *et al*. 1998) and Ry_{sto} (Brigneti *et al*. 1997), the potato cyst nematode

resistance QTL *Gro1.3* (Kreike *et al*. 1993) and the potato leafroll virus resistance QTL PLRV.1 (Marczewski *et al.* 2001), are localised on this distal part of chromosome 11. Other resistance gene clusters in potato are found on chromosome 5 and 12 (Gebhardt & Valkonen 2001). Most of the cloned resistance genes contain similar components involved in the recognition system of the plant. Based on this homology, it is hypothesised that the genes are evolutionary related and restricted to a few loci on the genome. The clustered resistance genes may be evolved by gene duplications followed by structural and functional diversification. This diversification can lead to gene clusters containing homologous genes acting to distinct pathogen species, like the two resistance genes of the gene cluster on chromosome 12, that give resistance towards potato virus X and the potato cyst nematode *G. pallida* (Van der Vossen *et al*. 2000). It is therefore not unlikely that the mapped $R_{Mc1\text{-}fen}$ gene is homologous to other resistance genes in the cluster on chromosome 11.

Chapter 6

Mapping and comparative analysis of the chromosome *11* root-knot nematode (*Meloidogyne chitwoodi*) resistance gene $R_{Mc1\text{-}hou}$ from wild potato (*Solanum hougasii*).

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Abstract

The resistance to the root-knot nematodes *Meloidogyne chitwoodi* and *M. fallax* present in the hexaploid wild potato species *Solanum hougasii* was efficiently mapped using bulked segregant analysis (BSA) in an inter-specific mapping population. The BSA, carried out with 394 AFLP primer combinations, resulted in a linkage group consisting of the resistance locus and 14 AFLP markers representing a genetic interval of 24 cM. The monogenic resistance locus $R_{Mc1\text{-}hou}$ is located at the distal end on the long arm of chromosome 11, a well known hotspot of resistance genes, among others the root-knot nematode resistance genes $R_{Mc1\text{-}blb}$ and $R_{Mc1\text{-}fen}$. The effect of the resistance gene introgressed from *S. hougasii* was compared with the other two mapped root-knot nematode resistance genes from *S. bulbocastanum* and *S. fendleri*. A comparative analysis of virulence and resistance was performed with *Solanum* genotypes carrying an R_{Mc1} gene and six nematode populations of *M. chitwoodi*. The tested nematode populations represented avirulent isolates from natural populations of race 1 and 2, a resistance breaking nematode population of race 2 formerly known as race 3, and a mono-female line selected from a successful infection event on *S. fendleri* by an avirulent population of race 1. The resistance breaking capacity of two virulent populations is confirmed. In the avirulent populations a low frequency of successful infection events is observed. The genotypes carrying an *R*-gene do not show clear differential interaction patterns with the nematode populations, suggesting a similar spectrum of resistance for $R_{Mc1\text{-}hou}$, $R_{Mc1\text{-}blb}$, and $R_{Mc1\text{-}fen}$.

Introduction

The potato (*Solanum tuberosum* L.) is like most modern crop plants vulnerable to a range of pests and diseases. One of the pests causing considerable losses in yield and quality is the Colombian root-knot nematode *Meloidogyne chitwoodi* (Golden *et al.* 1980). The nematode is found in different locations throughout the western states of the USA (Evans & Trudgill 1992, Santo *et al* 1980, Szalanski *et al* 2001), Mexico, Portugal, South Africa, the Netherlands and Belgium (summarized in Van der Beek 1997). This nematode has a broad host range and is therefore difficult to control via crop rotation. Breeding for resistance is considered to be an effective and environmentally safe strategy to control this nematode.

Within the cultivated potato gene pool only susceptible genotypes are found. Several wild *Solanum* species have been identified as valuable sources for the introgression of resistance genes. Among these are *S. bulbocastanum* Dun., *S. hougasii* Corr. and *S. fendleri* A.Gray, of which the resistance genes perform as a monogenic and dominant trait (Brown *et al.* 1996, 1999; Janssen *et al.* 1998; Rouppe van der Voort *et al*. 1999). So far, two resistance genes have been mapped: $R_{Mc1\text{-}blb}$ of *S. bulbocastanum* (Brown *et al.* 1996, Rouppe van der Voort *et al*. 1999), and $R_{Mc1\text{-}fen}$ of *S. fendleri* (Chapter 5). Both resistance genes mapped on a similar distal map position on the long arm of chromosome 11. The resistance genes in these species share, besides their map position, also another feature. Both appeared to be effective to the false root-knot nematode *M. fallax* (Janssen *et al.* 1997c, Rouppe van der Voort *et al.* 1999, Chapter 5). *M. fallax* is a species genetically closely related to *M. chitwoodi* (Karssen 1996).

Besides the Netherlands, the presence of this nematode species is also documented in France (Daher *et al.* 1996) and New Zealand (Marshall *et al.* 2001).

At present two races of *M. chitwoodi* are recognised. The basic difference between race 1 and race 2 resides in their reproduction on the alfalfa cv. Thor and the carrot cv. Red Cored Chantenay. *M. chitwoodi* race 2 reproduces on alfalfa but not on carrot. Conversely, alfalfa is a poor host and carrots are suitable for *M. chitwoodi* race 1 (Santo & Pinkterton 1985, Mojtahedi *et al.* 1988). A virulent biotype of race 2, previously separately indicated as a race 3, is able to overcome the resistance in *S. bulbocastanum* (Mojtahedi *et al.* 1994). In a study with eight different *M. chitwoodi* populations of race 1 and five *Solanum* species, no significant interaction was found between the plant genotypes and nematode populations, suggesting little variation in virulence factors across sampled populations of race 1 (Van der Beek *et al.* 1998b). However, within each population multiple virulence factors could be present at variable allele frequencies. In a greenhouse experiment Janssen *et al*. (1998) succeeded in the selection of mono-female *M. chitwoodi* lines with increased virulence that could overcome the resistance gene $R_{Mc1\text{-}fen}$ by multiplying egg-masses which are occasionally formed on a resistant genotype. Most likely this virulence factor was already present at a low allele frequency in the largely avirulent population.

In this paper we describe the genetic localisation of the resistance gene $R_{Mc1\text{-}hou}$ present in *S. hougasii*. In addition, the spectrum of this resistance gene was analysed using a series of isolates of *M. chitwoodi*. Finally, the resistance spectrum of the current $R_{Mc1\text{-}hou}$ is compared with the resistance genes $R_{Mc1\text{-}blb}$ and $R_{Mc1\text{-}fen}$ to allow discussion on the value of these genes in breeding.

Materials & Methods

Plant material

The pedigree of the mapping population is presented in Figure 1. The genotype M94-110-2 is a pentaploid inter-specific hybrid from a cross between the hexaploid *S. hougasii* BGRC55203 and a tetraploid *S. tuberosum* breeding line. This *S. hougasii* genotype is resistant to *M. chitwoodi* and *M. fallax* (Janssen *et al.* 1997c). Backcrosses were made to assure segregation and a simplex inheritance of monogenic resistance. On the basis of its high resistance and agronomic value the BC1 clone RH4X-029-2 was chosen as the resistant parent for a BC2 mapping population.

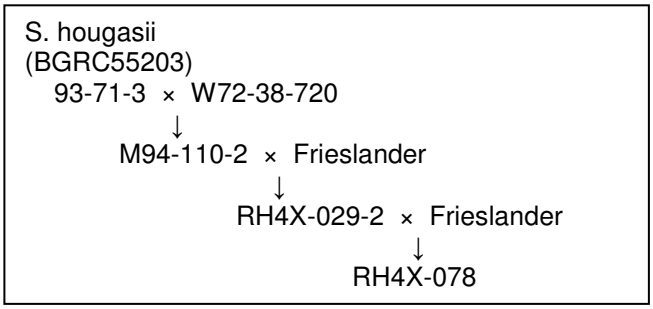

Figure 1. Crossing scheme of the mapping population RH4X-078

Seeds of the RH4X-078 population were sown in vitro on a MS30 medium (Murashige & Skooge 1962). After germination, 111 seedlings were multiplied in vitro to obtain a sufficient number of plants for replicated resistance assays, DNA extraction, and genotype maintenance. In vitro grown parents of the mapping population and the susceptible control variety Bintje were included in the resistance test. Three weeks after sub-culturing the plant material, the rooted cuttings were transplanted in 350 cm^3 clay pots containing silver sand and slow release NPK fertiliser ('Osmocote', Sierra Chemical Company, Milpitas, USA). The pots were placed in a greenhouse in a completely randomised design with initially 4 replicates of each clone. The temperature in the greenhouse ranged between 18° and 25°C. During the first six days after transplanting the relative humidity was kept near 100%. Four weeks after transplanting, the plants grew vigorously, and were inoculated with 1 ml of a water suspension containing approximately 500 juvenile (J2) larvae. All plants were tested for their level of resistance against *M. chitwoodi* population Co (formally known as CHE, Janssen *et al.* 1998) and *M. fallax* Fa (formally known as CHB, Janssen *et al.* 1998). Eight weeks after inoculation, the root systems of the plants were harvested, and rinsed to remove the sand, and stained with Phloxine-B (Dickson & Struble 1965). The clear difference between the amount of egg masses on resistant and susceptible genotypes allowed classification of the offspring genotypes. Besides of this classification, the number of egg-masses formed on the parents, controls and resistant plants were counted. The size of the root systems was recorded and plants with a small root system were excluded from further analysis.

Molecular analysis

Three in vitro cuttings per genotype were used for maintenance and DNA isolation. DNA isolation was performed as described by Van der Beek *et al.* (1992). The AFLP technology was carried out basically as described by Vos *et al.* (1995). Nomenclature of the AFLP-markers is based on the template enzyme combination abbreviated to one letter, followed by letters that represent the selective nucleotides, and a number describing the visually estimated mobility of the fragment relative to the bands of the SequaMark 10 base ladder (Research Genetics, Huntsville, AL, USA). The Bulked Segregant Analysis (BSA) method (Michelmore *et al.* 1991) was applied to identify linked markers. A set of eight susceptible plants and a set of eight resistant plants were used to compose the susceptible and resistant bulk. For this purpose we mixed equal aliquots of secondary template (restriction-ligation product amplified with pre-selective primers or E+A, M+C). BSA-AFLP was performed using 138 combinations of *Eco*RI+ANN primers with *Mse*I+NNN primers. A second round of BSA was performed using all 16 *Pst*I+NN × 16 *Mse*I+CNN primer combinations. These 256 primer combinations were performed on one resistant bulk and two susceptible bulks, each comprising eight individuals. Primer combinations showing bulk specific fragments were verified on separate individuals of the entire mapping population (or a subset) to confirm genetic linkage with the resistance gene. The genetic data were analysed using JoinMap version 2.0 (Stam & Van Ooijen, 1995).

In order to assign the correct chromosome number to the linkage group, the strategy of Rouppe van der Voort *et al.* (1997b) was followed. This strategy implies that AFLP markers with equal mobility on gel, are most likely to show DNA sequence homology and represent the same locus

on a linkage map. Therefore the reference genotypes C, E, AM, RH and SH were also included during analyses of the primer combinations that showed a bulk specific AFLP marker. In the case co-migrating markers were present in any reference genotype, the map position of the marker could be retrieved from a database (Rouppe van der Voort *et al.* 1998a; http//:www.dpw.wur.nl/pv/aflp/catalog.htm). To confirm the identity of the linkage group, the CAPS marker M39b (Figure 2) was analysed using PCR-conditions according to Brigneti *et al.* (1997). The PCR-product was digested with the restriction enzyme *DdeI* (Brigneti *et al*. 1997).

Comparative analysis of virulence and resistance spectrum

The effect of the resistance gene introgressed from *S. hougasii* was compared with resistance genes from *S. bulbocastanum* and *S. fendleri*. Plant material carrying the resistance genes from the three potato species was analysed with six nematode populations of *M. chitwoodi* listed in Table 1. The populations Ca, Cat, Cba and Cbd represent natural populations that were propagated without selection. The isolate Cbh is a resistance breaking nematode population, which was formerly known as race 3 (Mojtahedi *et al.* 1994). The isolate Ccl represents a mono-female line that was selected from a successful infection event on *S. fendleri* by the avirulent population Co (formerly known as population CHE; Janssen *et al.* 1998). The bioassay was performed in 4 replications as described above, but the amount of newly formed egg-masses was counted to allow statistical analysis of the interactions between nematode populations and potato genotypes. The potato genotypes used for this comparative analysis were the BC3-plants 95-B3-02, 95-B3-03 and 95-B3-13R derived from *S. bulbocastanum* by a cross between 398.89 × A84118.3 that was used to map the resistance gene $R_{Mc1\text{-}blb}$ on chromosome 11 (Rouppe van der Voort *et al.* 1999). An additional BC3-plant of *S. bulbocastanum*: 95-B5-07 was derived from a cross between 398.89 × Ranger Russet. The genotype M94-125-1 represents the resistant parent of a *S. fendleri* derived mapping population that was used to localise $R_{Mc1\text{-}fen}$ (Chapter 5). The resistant parent RH4X-029-2 of the *S. hougasii* mapping population, used in the present study, and the *S. tuberosum* cultivars Gloria (1963) and Bintje were added in the test as resistant and susceptible controls, respectively.

Table 1. Isolates of *M. chitwoodi* used in the virulence test.

Isolate	Race	Origin	Reference
Ca	1	NL	Van der Beek *et al.* (1999)
Cat	1	USA	Van der Beek *et al.* (1997)
Cba	1	USA	Van der Beek *et al.* (1999)
Cbd	2	USA	Van der Beek *et al.* (1999)
Cbh[1]	2	USA	Van der Beek *et al.* (1999)
Ccl[2]	1	NL	Janssen *et al*. (1998)

[1] a resistance-breaking biotype which was formerly known as race 3 (Mojtahedi *et al.* 1994)
[2] virulent line from isolate Co (formerly known as CHE)

Results

Resistance in the S. hougasii mapping population

The level of resistance across offspring genotypes did not fit a continuous distribution, but fell apart in two clearly distinguishable classes. Plants, which could be classified as resistant, showed between 0 and 4 newly formed egg-masses; on plants classified as susceptible the estimated amount of egg-masses ranged from 40 to 350. The high amount of egg-masses that developed on the susceptible progeny was similar to the amount of egg-masses formed on the susceptible control Bintje. Because of a poorly developed root system 7.1 % of the tested plants were excluded for further analysis. The nematode assay revealed a clear segregation of 56 susceptible and 53 resistant plants in mapping population RH4X-078, fitting the 1:1 Mendelian ratio (χ^2= 0.08) of a monogenic trait. Therefore we conclude the presence of a monogenic resistance locus, named $R_{Mc1\text{-}hou}$, according to the gene nomenclature proposed by Brown *et al*. (1999). The average number of egg-masses formed by *M. chitwoodi* and *M. fallax* on the parents, on the susceptible control and on the resistant progeny is listed in Table 2. The maximum number of egg-masses that was observed on resistant clones was four and three egg-masses by *M. chitwoodi* and *M. fallax*, respectively. The segregation of the resistance against *M. chitwoodi* and *M. fallax* was completely correlated.

Table 2. Mean amount of egg-masses of *M. chitwoodi* and *M. fallax* found on the parents of the mapping populations, the resistant genotypes of the mapping population and on the susceptible control Bintje.

	RH4X-029-02	Frieslander	Resistant progeny	Bintje
M. chitwoodi	0.3	23.3	0.2	108.3
M. fallax	0.0	27.0	0.1	105.3

AFLP-analysis of the S. hougasii mapping population

Resistant and susceptible bulks were composed from eight offspring genotypes randomly chosen from the resistant and susceptible class. The parents and bulks were screened with a total of 394 AFLP primer combinations (138 EcoRI/MseI and 256 PstI/MseI). Bulk specific AFLP bands, showing an AFLP-fragment present in the resistant bulk only, were observed in 30 primer combinations. Bulk specificity was verified using the separate offspring templates. Fourteen of the thirty AFLP-fragments showed genetic linkage with resistance. This provides a further confirmation of the monogenic character of this resistance. The linkage map comprising these 14 AFLP markers represents a genetic interval of 24 cM (Figure 2).

The strategy of Rouppe van der Voort *et al.* (1997b) to determine the chromosomal identity of the linkage group was not successful. None of the scored AFLP-markers was present among the reference genotypes of the AFLP-catalogue (Rouppe van der Voort *et al.* 1998a). However one co-migrating AFLP marker was detected in another mapping population RH4X-088 (Chapter 5). The resistant parent RH4X-036-11 of this mapping population contains the $R_{Mc1\text{-}fen}$ resistance gene. The $R_{Mc1\text{-}fen}$ resistance gene was mapped at the distal position on the long arm of chromosome 11 and showed no recombination with the AFLP marker P+GG/M+CAC_302.

This AFLP marker was the same as the marker that mapped most close to the resistance gene in the *S. hougasii* mapping population at a distance of 1 cM (one recombination event). This result demonstrated once more the locus-specificity of AFLP markers and the utility of co-migrating markers to detect the chromosomal identity of a linkage group. Independent confirmation was obtained with the chromosome 11 specific CAPS marker M39b, that maps to $R_{y\text{-}sto}$ and $R_{Mc1\text{-}blb}$ (Brigneti *et al*. 1997, Rouppe van der Voort *et al.* 1999). This CAPS marker mapped in the *S. hougasii* mapping population at a distance of 2 cM (two recombination events) from the $R_{Mc1\text{-}hou}$ resistance gene (Figure 2).

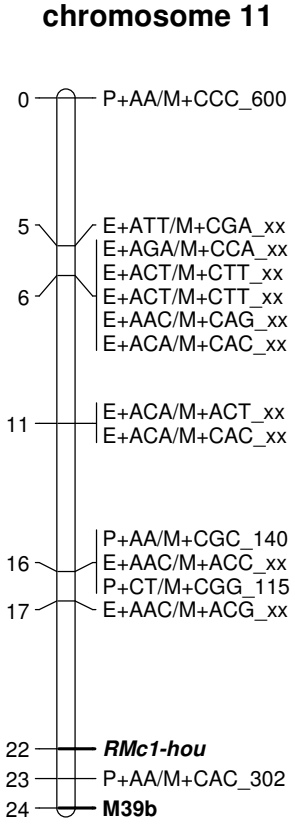

Figure 2: Position of the $R_{Mc1\text{-}hou}$ locus on the long arm of chromosome 11 of the resistant clone RH4X-078. Chromosome orientation is according to Dong *et al.* (2000). AFLP-markers of which the sizes are not estimated are marked by xx.

Comparative analysis of the resistance spectrum of R_{Mc1}-genes

Eight *Solanum* genotypes have been tested for their level of resistance against six root-knot nematode populations. The average number of the newly formed egg-masses observed in this assay is listed in Table 3. The large differences in the number of egg-masses provide clear evidence for the absence and presence of *R*-genes in potato genotypes, as well as the absence and presence of specific avirulence factor(s) in the nematode populations. The *S. tuberosum* clones do not carry effective *R*-genes, since large numbers of egg-masses are formed. The

genotypes that carry an *R*-gene do not show clear differential interaction patterns with the nematode populations, suggesting a similar spectrum of resistance for $R_{Mc1\text{-}hou}$, $R_{Mc1\text{-}blb}$, and $R_{Mc1\text{-}fen}$.

The resistance breaking capacity of population Cbh and Ccl is confirmed. These populations produce egg-masses on material with an *R*-gene at a level that equals susceptible *S. tuberosum*. In the avirulent populations Ca, Cat, Cba and Cbd a low frequency of successful infection events is observed. For Cbd the rate of successful infections is considerably higher as compared with the avirulent populations Ca, Cat and Cba. Remarkably, the more virulent population Cbd did not produce egg masses on the genotypes 95-B3-03 and 95-B3-13R that carry $R_{Mc1\text{-}blb}$. Within the group of six resistant potato genotypes analysis of variance showed a significant effect of the potato genotype on the number of egg masses produced by Cbd ($p=0.019$). No significant effect of potato genotype on egg mass production was observed for other nematode populations (data not shown), because of the highly variable number of egg masses between replications and variation in root system development.

Table 3. Average number of newly formed egg-masses on eight *Solanum* genotypes using six different root-knot nematode populations.

***Solanum* spp. and genotype**	***R*-gene**	**Root-knot nematode population**						
		Ca race 1 NL	**Cat** race 1 NL	**Cba** race 1 USA	**Cbd** race 2 USA	**Cbh**[1] race 2 USA	**Co**[2] **(CHE)** race 1 NL	**Ccl**[1] race 1 NL
S. tuberosum								
Bintje	None	51.8	140.0	135.5	134.5	110.0	susceptible	171.5
Gloria	None	100.8	75.8	24.3	49.0	109.3	susceptible	56.3
S. bulbocastanum								
95-B3-02	$R_{Mc1\text{-}blb}$	0.0	0.0	1.0	5.3	97.3	resistant	97.0
95-B3-03	$R_{Mc1\text{-}blb}$	0.0	2.0	0.7	0.0	67.0	resistant	153.5
95-B3-13R	$R_{Mc1\text{-}blb}$	0.0	0.0	0.3	0.0	90.8	resistant	123.8
95-B5-07	$R_{Mc1\text{-}blb}$	0.0	0.0	0.3	5.7	47.3	resistant	227.3
S. fendleri								
M94-125-1	$R_{Mc1\text{-}fen}$	0.3	0.0	0.3	15.0	84.0	resistant	103.5
S. hougasii								
RH4X-29-2	$R_{Mc1\text{-}hou}$	0.0	0.0	1.0	5.0	82.5	resistant	28.5

1) The population Cbh (also known as CAMc2) was formerly regarded as an USA race 3 population because of its ability to multiply on a resistant *S. bulbocastanum* clone (Mojtahedi *et al*. 1994). Population Ccl represents a mono-female line that was selected from an occasional infection event on *S. fendleri* of population Co (Janssen *et al*. 1998).

2) The amount of inoculum of population Co that could be reared was insufficient to allow accurate testing. The results for Co were added for the sake of comparison, and are based on previous mapping experiments (see text). The resistant plants occasionally formed egg masses.

Discussion

The mapping of the $R_{Mc1\text{-}hou}$ gene involved in resistance against M. chitwoodi (race 1) and M. fallax on potato chromosome 11

In this paper the mapping of the *S. hougasii* derived $R_{Mc1\text{-}hou}$ gene is described. The straightforward introgression of the gene into the cultivated potato via repeated backcrosses and the simple inheritance of the resistance phenotype clearly show that the resistance to *M. chitwoodi* race 1 is a monogenic and dominantly inherited trait. Brown *et al*. (1999) also described the presence of such resistance factors in *S. hougasii*, and proposed a nomenclature for these *R*-genes. At this moment the name $R_{Mc1\text{-}hou}$ offers an adequate description, although allelism to the *R*-gene described by Brown *et al*. (1999) is not proven. In future it may be required to add more suffixes to the name, such as $R_{Mc1\text{-}hou}$*11.1* to indicate also the map position on chromosome *11* and a serial number, if additional genes are localised on this chromosome.

The $R_{Mc1\text{-}hou}$ locus was not only effective to *M. chitwoodi* population Co, but also against a *M. fallax* population. This combined resistance to *M. fallax* and *M. chitwoodi* has been described before for the resistance gene $R_{mc1\text{-}blb}$ of *S. bulbocastanum* (Janssen *et al.* 1997a, Rouppe van der Voort *et al.* 1999) and $R_{mc1\text{-}fen}$ of *S. fendleri* (Chapter 5). This could indicate that a single gene at this locus can recognise similar or identical avirulence gene products of *M. chitwoodi* and *M. fallax*. Alternatively, this locus could contain two closely linked *R*-genes, which is not uncommon since *R*-genes are frequently organised in clusters. It is not inconceivable that co-evolution between host and parasite resulted in divergence of the virulence factors which is matched by gene duplication in the *R*-gene cluster and co-divergence of the duplicated *R*-genes. When future research will reveal the existence of an individual *R*-gene for *M. fallax*, it should be named $R_{Mf\text{-}hou}$.

The *S. hougasii* derived mapping population RH4X-078 was analysed only with *M. chitwoodi* population Co, whereas the resistant parent also resisted the populations Ca, Cat and Cba (race 1), and the population Cbd (race 2). Therefore it is tempting to speculate on the presence, segregation and localisation of other resistance genes present in this mapping population. Two arguments plead against this. First, the independent work on three taxonomically distinct potato species did not result in the detection of more than one locus. Second, it did not result in the detection of differential gene interaction between the R_{mc1} genes and different nematode populations, including race 1 and race 2, and resistance breaking mono-female lines.

The resistance gene $R_{Mc1\text{-}hou}$ was mapped relative to 14 AFLP markers which were obtained using bulked segregant analysis of 394 AFLP primer combinations. In view of theoretical expectations on the efficiency of BSA in tetraploid potato (Li *et al*. 1998) the rate of 14 out of 394 should be regarded as highly successful, and comparable to their rate of 11 markers out of 205 primer combinations. Elevated DNA sequence dissimilarity between *S. hougasii* and *S. tuberosum* could be responsible for this success rate. The R-gene is located at the distal end of the linkage group, and fortunately enough there are flanking markers allowing applications such as marker-assisted selection and map based cloning.

The alignment of the linkage group to chromosome *11*, using the strategy of co-migrating AFLP markers, was not successful when using *S. tuberosum* related reference material. However, when *S. hougasii* fingerprints were compared with fingerprints from a *S. fendleri* derived mapping population, the correct linkage group could be assigned. Earlier statements about the utility of a catalogue of co-migrating AFLP fragments (Rouppe van der Voort et al., 1998a) made clear that reference material should represent germplasm with a sufficient degree of fingerprint similarity. Apparently, at this locus, *S. fendleri* (series *LONGIPEDICELLATA* Buk.) and *S. hougasi* (ser. *DEMISSA* Buk.) are more similar to each other as compared to cultivated potato (ser. *TUBEROSA* Hawkes). The AFLP marker P+GG/M+CAC_302 that mapped near the resistance gene $R_{Mc1\text{-}hou}$, was also polymorphic in the *S. fendleri* mapping population were it co-segregated with the resistance gene $R_{Mc1\text{-}fen}$ on chromosome *11* (Chapter 5). An additional CAPS marker M39b confirmed the chromosome number of the linkage group. This marker mapped at a distance of 2 cM to the resistance gene, a distance that is equal to the distance between this marker and the *M. chitwoodi* resistance gene $R_{Mc1\text{-}blb}$ from *S. bulbocastanum* (Rouppe van der Voort *et al.* 1999).

All three R_{Mc1} genes appeared to localise in the same distal region of the long arm of chromosome 11. This region is a well known hotspot of resistance genes, including the white potato cyst nematode resistance gene $R_{Gpa\text{-}tar}$ (Wolters *et al.* 1998), the potato virus Y resistance genes Ry_{sto}, Ry_{adg} and Na_{adg} (Hämäläinen *et al.* 1997; Brigneti *et al.* 1997; Hämäläinen *et al.* 2000), and the wart disease resistance gene *Sen1* (Hehl *et al.* 1999). Such numbers of resistance genes residing in such a small region on the genome, will severely complicate the pyramiding of resistance genes. The selection of valuable recombinants, which carry multiple resistance genes in coupling phase, should be pursued with the assistance of DNA markers.

Comparative analysis of the resistance spectrum of R_{Mc1}-genes

From the potato breeding perspective the value of resistance genes is defined by their ability to avoid yield reduction and to prevent the propagation of pathogen inoculum as well as by the spectrum of isolates they give resistance to. To investigate the spectrum width the $R_{Mc1\text{-}hou}$ gene was tested against six root-knot nematode populations. The gene does not confer broad-spectrum resistance against all populations of *M. chitwoodi*, because race 1 isolate Cbh and race 2 isolate Ccl could produce egg-masses. The number of populations that are available for *M. fallax* is too limited to allow generalisations on broad-spectrum resistance of $R_{Mc1\text{-}hou}$ against *M. fallax*.

When the spectrum of the $R_{Mc1\text{-}hou}$ gene is compared with that of the genes $R_{Mc1\text{-}fen}$ and $R_{Mc1\text{-}blb}$, it is clear that all genes have a similar effect on the six nematode populations. In view of this result it does not seem advantageous to potato breeders to start pyramiding the $R_{Mc1\text{-}hou}$, $R_{Mc1\text{-}fen}$ and $R_{Mc1\text{-}blb}$ genes. Further research is needed to find out if combinations of these three genes could have a synergistic effect, and recognise different virulence factors. Alternatively, the cloning of these genes will allow the identification of putative differences between these genes at the level of the DNA sequence. In view of the strong conservation of *R*-gene sequences

within *R*-gene clusters (Bakker 2003) strong homology could be expected, in spite of the large taxonomic distances between the series *BULBOCASTANA* (Rydb.) Hawkes, *LONGIPEDICELLATA* Buk., and *DEMISSA* Buk. from which *S. bulbocastanum,S. fendleri* and *S. hougasii* are members.

Comparative analysis of nematode populations

A series of six nematode populations was analysed with susceptible potato and R_{mc1} genes from three different Solanum species. Difference in virulence between race 1 and race 2 was not found between the races but within. Both within race 1 and race 2 populations were available (Ccl and Cbh) that could produce egg-masses. The population Cbh (also known as CAMc2) has been described as a resistant breaking race 2 population. It was able to multiply on the *S. bulbocastanum* genotype SB22 (Mojtahedi *et al.* 1994). We showed that Cbh could overcome the resistance of all three wild species.

Populations Ccl and Cbh seem to have a similar virulence spectrum, although they have been described initially as race 1 and race 2, respectively. Van der Beek *et al.* (1998b) tested eight Dutch *M. chitwoodi* populations with the differentials to distinguish race 1 from race 2. They found no indication for race 2 in the Netherlands. Population Ccl has not yet been tested on alfalfa cv. Thor to exclude the possibility that a small fraction of the individuals in population Co belongs to race 2 (Ccl is a mono-female isolate derived from Co, Janssen *et al*. 1998).

On the basis of the data summarised in Table 3 we have not been able to detect significant interaction between resistant potato genotypes and nematode populations, except for *S. bulbocastanum* genotypes with a significant different response to Cbd. One possible interpretation could be the presence of a low fraction of resistance breaking individuals in Cbd, and the presence of an additional factor involved in resistance in genotypes 95-B3-03 and 95-B3-13R.

At this moment two virulent populations Cbh and Ccl are available. Population Cbh is a natural population and Ccl is an artificial isolate selected as a mono-female line from egg-masses that are occasionally formed on resistant genotypes upon inoculation with a natural population.

Also in the *M. fallax* population, occasionally egg-masses are formed on resistant plants, but these egg-masses have not been reared to test the ability of *M. fallax* to generate virulence breaking mono-female populations. The data from Table 3 show that all avirulent populations Ca, Cat, Cba and Cbd could form egg-masses in low amounts. The same phenomenon was observed for the population Co that was commonly used for the mapping of resistance in *S. bulbocastanum* (Rouppe van der Voort *et al.* 1999), *S. fendleri* (Chapter 5), and *S. hougasii* (this study).

Durability of the $R_{Mc1\text{-}hou}$, $R_{Mc1\text{-}fen}$ and $R_{Mc1\text{-}blb}$ resistance genes

The occasionally formed egg-masses may have great implications for the durability of the R_{Mc1} genes. Possibly, those egg-masses result from an avirulent nematode that escaped the

resistance response in the plant. For several plant-pathogen systems incomplete expression of resistance has been described. A more plausible explanation for the occasional production of egg-masses by Ca, Cat and Cba is the presence of a small fraction of virulent individuals, in analogy with the origin of Cbh and Ccl.

Without crop rotation, the fraction of virulent nematodes in a population will increase dramatically, due to the strong selection imposed by resistant cultivars. However, selection imposed by alternative (resistant) hosts, grown in the years without potato, could reduce these effects on population allele frequencies. Cyclical selection can result in trajectories of gene frequencies that fluctuate around an equilibrium point (Leonard 1977).

In addition to forecast the durability of *R*-genes, the development of mono-female lines is an important tool for both the categorisation of the different virulence factors in *M. chitwoodi* populations and the identification of R_{Mc1} genes in wild *Solanum* species. In this study we used the virulent populations Cbh and Ccl to screen potato genotypes. We observed that these two populations were able to circumvent the resistance present in *S. fendleri*, *S. hougasii* and *S. bulbocastanum* derived breeding lines, confirming the results of Janssen *et al*. (1999).

Chapter 7

General Discussion

Introduction

The aim of the research described in this thesis is the development and implementation of molecular markers in potato breeding. Although molecular marker development is ongoing for many years, the practical use in Marker Assisted Selection (MAS) in potato breeding is rare. Our study is not just another attempt to map resistance loci but also an attempt to implement MAS in a potato breeding programme. The ultimate goal is the introduction of potato cultivars with resistance to the root-knot nematodes *Meloidogyne hapla*, *M. fallax* and *M. chitwoodi*, selected with molecular markers. In the current potato cultivars resistance to these nematodes is not available. The results described here will contribute to a more rapid development of resistant potato cultivars. The selection in breeding material based on genotype instead of phenotype and selection in seedling stage are ultimate tools for a plant breeder as will be discussed in this chapter. MAS will not replace classical breeding but it will become a tool for the selection at an earlier stage, eventually cheaper and providing new opportunities in resistance breeding by pyramiding genes. The strategy followed in marker development, the durability of the resistance and the consequences of the implementation of molecular markers in potato breeding, will be discussed.

Potato breeding

The most important characters in potato breeding are a high yield combined with good quality. The quality depends on the different breeding objectives like French fries, chips, fresh consumption and starch potatoes. Within these objectives the requirements of quality can also depend on local preferences that differ per country. Besides the quality and other agronomical traits, resistances to diverse pathogens and pests are important. Selection for resistances and agronomical traits are almost always made based on the phenotype of the clones. The potato clones are, therefore, tested in the field for the agronomical traits and in bioassays in field and laboratory for the different diseases and pests. In some area's resistance to certain pests are a prerequisite like wart disease and potato cyst nematode resistance in starch potato cultivars grown in the north east of The Netherlands. Therefore the first bioassays are already performed after two years of selection. Because the amount of produced seed potatoes is limited, the bioassays will then be performed in just one replication. Especially the two mentioned traits are very sensitive to the environment, which implies that more replications should be made. These resistance tests will therefore be repeated the next years in the breeding programme with every year in succession more replications. Because the large amount of tests the breeding of a new potato cultivar is a slow process. It takes approximately 9 to 12 years from making the cross to the admission for the list of varieties. New techniques to speed up the breeding process are available but not yet fully implemented into the breeding process. One of these techniques is marker assisted selection (MAS). The molecular markers which are diagnostic for certain traits are identified by genetic analysis of segregating populations. These molecular markers can eventually lead to the cloning of genes and subsequently the genetic modification of a variety. Genetically modified varieties with an enhanced resistance to diseases and pests should be an acceptable solution to reduce the chemical input in agricultural production. Regrettably due to the opposition of certain environmental groups the GMO approach in potato has been limited in The Netherlands to one potato with a modified starch composition to be used for industrial

purposes. Until the time genetic modification of plants will be accepted, the potato breeders have to incorporate resistance in potato cultivars by means of traditional breeding methods. In order to speed up the resistance breeding, they can use MAS.

Resistance breeding in potato

The first step in resistance breeding is the identification of genetic variation within potato cultivars and within its wild relatives. Sufficient genetic diversity is available in the *Solanum* species including resistances to diseases and pests that can be used for the introgression of resistance into potato cultivars. Many of these wild potato species have a diploid chromosome level, in contrast to the common potato cultivars that have a tetraploid level. The use of a diploid potato breeding programme allows direct gene transfer from these wild diploid *Solanum* species to *S. tuberosum*. An additional advantage of breeding at the diploid level is the more efficient backcrossing process, necessary after such an intraspecific cross. At the diploid level the offspring will also display a wider spectrum of phenotypic variation, which implicates a smaller progeny size and a lower number of backcrosses needed. Besides the more efficient breeding, the use of diploid potatoes simplifies the genetic research compared with tetraploid potato, specifically when multiple genes are involved in the character studied. In the research described in this thesis polyploid as well as diploid wild species are used.

We followed the strategy of combining introgression breeding and genetic research. The genetic research should reveal the loci of the resistance genes. After developing closely linked markers, MAS can be applied to speed up the breeding process. Resistant hybrids are used for further crossing with potato clones and selection on agronomical traits will be done phenotypically on the resistant progeny of these crossings. Although interspecific backcrosses can be complex due to crossings barriers, the application of this strategy will lead to the introgression of the resistance which is a goal on its own. Moreover, when polyploid wild species are used, interspecific backcrosses will lead to a simplex/nulliplex condition of the target gene, as well as for the markers, as required for BSA. In Chapter 5 two strategies are compared by generating both an intraspecific and interspecific mapping population for the detection of resistance loci. Although we have a limited amount of data, the results support the commonly observed reduced level of recombination between the introgression segment and the recipient genome of interspecific introgression material (De Vincente & Tanksley 1991; Ganal & Tanksley 1996). In the same chapter we observed an elevated level of polymorphisms in the interspecific mapping population. This can be due to the fact that *S. fendleri* is a self-fertilising species, so in an intraspecific mapping population an elevated level of homozygosity is expected. Additionally the dissimilarity between the wild species introgression segments and the recurrent potato parents in the interspecific mapping population may have caused an elevated level of polymorphic markers.

Root-knot nematode

After *P. infestans* the second important pest in Western Europe is the potato cyst nematode *Globodera rostochiensis* and *G. pallida*. Potato breeders have been very successful in breeding for resistance to *G. rostochiensis*. Most of the Dutch potato starch cultivars and a few consumption potato cultivars are resistant to *G. pallida*. When farmers choose resistant

cultivars they should be able to control the potato cyst nematode problem. This choice should be based on the identity of the resistance gene and the avirulence of the nematode population. A good variety management will eventually lead to a more cost efficient and environmentally safe way of producing potatoes. The use of resistant cultivars already led to a substantial reduction in chemical use in the intensive potato cultivation. This has however also led to an increasing problem with other nematodes like *Pratylenchus penetrans*, *P. crenatus, Trichodorus* spp. and the root-knot nematodes *Meloidogyne chitwoodi*, *M. fallax* and *M. hapla*. The most abundant of these nematodes are the *Pratylenchus* and *Trichodorus* species. The root-knot nematodes are however also an increasing problem, especially in the southern part of The Netherlands where almost 25% of the investigated soil samples of the fields with assumed nematode problems, contained among others *M. chitwoodi* nematodes (pers. com. J. Peltjes, HLB). The sandy soils and the intensive cultivation of potatoes also makes the circumstances in the north east of The Netherlands favourable for the increase and spread of *M. chitwoodi* and *M. fallax*. In contrast to nematodes like *Pratylenchus* spp. and *Trichodorus* spp., the root-knot nematode can be transferred with the seed potatoes. The risk of spreading the root-knot nematodes by seed potatoes is especially large when the production of seed potatoes is based on the regulation of farmer saved seed. This method of seed production has a minimal phytosanitary control by the government and is therefore an important factor in the spread and increase of the root-knot nematodes. In addition most of the potatoes grown in the starch producing area are late maturing varieties, which offer the nematodes the possibility of more generations and therefore a larger increase of the population. Under favourable conditions *M. chitwoodi* can have up to 4 generations per growing season (Pinkerton *et al.* 1991). The problems with *M. hapla* in the potato cultivation seem less critical as compared with *M. chitwoodi* and *M. fallax*. The occurence in The Netherlands of *M. hapla* is less frequent than the other two root-knot nematodes. In contrast to the swelling or galls and discoloration of the flesh caused by *M. chitwoodi* and *M. fallax*, the damage caused by *M. hapla* is limited to yield loss. This reduction in yield is however not always recognized by the farmer as a result of *M. hapla* infestation. In areas where potato is part of a crop rotation with crops like carrot, pea, black salsify, onion or chicory, *M. hapla* may cause more problems, due to damage in the follow up crop after the increase of the nematode population during the growth of potato.

Strategies for controlling the root-knot nematode

The control of the root-knot nematodes by means of chemical fumigation is an effective and economically feasible method but from an environment point of view undesirable. An alternative method of control is the use of resistant green manure crops, like winter radish. It is known that there are differences in multiplication among varieties of this crop but more research is required to assess the economical feasibility of this method. Another possibility to reduce the damage of root-knot nematodes is a wider crop rotation. Crop rotation however might be only effective to control *M. hapla* which does not multiply on monocotyledons. Cultivation of monocotyledons is most likely the reason why *M. hapla* is not frequently found in the north east of The Netherlands. The broad host range of *M. chitwoodi* and *M. fallax* makes crop rotation more complicated as they multiply on most of the crops used in common rotation schemes like sugar beet, wheat and barley. Looking at the larger problem with *M. chitwoodi* and *M. fallax*, the

potato growers are best served with resistant potato cultivars to these nematode species. The successful introgression of resistance to potato cyst nematodes can be taken as an example for the control of root-knot nematodes.

We have shown in Chapters 5 and 6 that the resistance is controlled by a single locus, effective to *M. chitwoodi* and *M. fallax*. This means that the resistance to both species is controlled by the same gene or that the resistance locus contains two closely linked *R*-genes. Both hypotheses are liable, since *R*-genes are frequently organised in gene clusters and examples of resistance genes acting to distinct pathogen species are known (Van der Vossen *et al.* 2000). In both cases the monogenic and dominant inheritance of the resistance provides the potato breeder the opportunity to develop resistant cultivars in a relative short period of time. Compared with *M. chitwoodi* and *M. fallax* the resistance to *M. hapla* is more difficult to incorporate into new cultivars. Although we did not always find more than one resistance gene it is evident that more factors play a role in the resistance to *M. hapla*.

Durability of resistance

In order to predict the durability of the resistance genes and to develop an appropriate breeding strategy it is important to have knowledge of the virulence present across nematode populations. Resistance breaking pathotypes are often a major concern in breeding programmes. In the resistance breeding against *P. infestans* this concern led to avoiding the introduction of major *R*-genes and to select for horizontal resistance only. The durability of resistance is depending on the resistance breaking ability of the pathogen and the presence of virulence in the population. Compared to many aerial pathogens the resistance breaking ability of potato cyst nematodes and root-knot nematodes is lower, because they are soil-borne and sedentary and therefore the rates of gene flow and spread of virulence alleles will be low. The durability of a major resistance gene against the potato cyst nematodes has been shown in the United Kingdom where potato cultivars with the *H1* resistance gene from *S. tuberosum* ssp. *andigena* have been widely grown over 30 years without selecting virulent populations so far (Turner & Flemming 2000). For the *Globodera* spp. it has been pointed out that the different pathotypes already existed in the initial nematode population in Europe. The use of resistance genes like the *H1*-gene in the breeding programme and subsequently in the cultivars, lead to a change of virulences in the population, more than only mutation in a virulent nematode (Folkertsma *et al.* 2001). Despite the shorter generation time of *Meloidogyne* spp., it is feasible that the root-knot nematode has a comparably poor resistance breaking ability as the potato cyst nematode. Consequently the durability of the resistance to the root-knot nematodes would also mainly depend on the variability in virulence of the population already present in the soil. In contrast to the potato cyst nematode of which only a small proportion of the nematode genepool has been introduced in Europe, the root-knot nematode populations are presumably not a result of a few small introductions into specific regions. When the virulence factors across nematode populations are known, the breeder is able to use effectively these populations to screen other wild *Solanum* species in order to detect other resistance genes.

Durability of the qualitative resistance to *M. chitwoodi* and *M. fallax*

In Chapters 5 and 6, we showed that occasionally egg-masses of *M. chitwoodi* and *M. fallax* were formed on the resistant genotypes of the mapping population. This has also been found in other studies (Janssen 1997a; van der Beek 1997; Austin *et al*. 1993; Brown *et al.* 1989,1991,1995). A possible explanation is that these egg-masses are a result of avirulent nematodes that escaped the resistance response in the plant. However, Janssen *et al.* (1998) showed that the nematode populations are a mixture of virulent and avirulent genotypes. The ease of selecting a virulent population of *M. chitwoodi* by Janssen *et al.* (1998) raise questions about the durability of the $R_{Mc1\text{-}fen}$ gene. Although their research was not performed in a field assay, their results may have great implications for the durability of all R_{Mc1} genes mapped in the experiments described in this thesis. The change of the allele frequency in time is influenced by 1) the meiotic parthenogenetic reproduction, 2) the multiplication and 3) the decrease during the growth of other crops. The shift in virulence might also retard when the fecundity of virulent isolates is lower, as has been shown in *M. incognita* (Castagnone-Sereno *et al.* 1994). The amount of produced egg masses of laboratory-selected virulent isolates of *M. incognita* differed between susceptible and *Mi*-resistant tomato clones, in contrast to virulent field isolates, suggesting a different genetic control of the virulence. We have not found indications that the fecundity of virulent isolates was different on susceptible plants compared with resistant plants (Chapter 6).

The variation in virulence in *M. chitwoodi* populations could also be demonstrated by the interaction to pepper lines containing *Me* resistance genes (Berthou *et al.* 2003). The Co population appeared to be avirulent to these *Me* resistance genes, similar to its interaction to the genes $R_{Mc1\text{-}fen}$ (Chapter 5), $R_{Mc1\text{-}hgs}$ and $R_{Mc1\text{-}blb}$ (Chapter 6). Two other Dutch *M. chitwoodi* populations showed virulence to the *Me* resistance genes and remarkably the *Me* resistant pepper was susceptible to the tested *M. fallax* population (Berthou *et al.* 2003). Our results did not show a differential interaction of *M. chitwoodi* and *M. fallax* to the three mapped R_{Mc1} genes (Chapters 5 & 6). The results of Berthou *et al.* (2003) and Chapter 6 show that more virulence factors may already be present in the Dutch *M. chitwoodi* populations. The amount of virulence factors present is however still unknown. Resistant cultivars can be used to select virulent mono-female lines. The different mono-female lines can subsequently be used to categorise the different virulence factors and give the breeder a tool for the selection of new sources of resistance in the wild *Solanum* species.

Durability of the quantitative resistance to *M. hapla*

The resistance to *M. hapla* described in Chapters 2 and 4 shows quantitative variation. For a long time quantitative resistance was considered to be race-non-specific, durable and equated with polygenic resistance. However in barley, cultivar by isolate interactions have been identified (Qi, 1998), and a ‘minor gene-for-minor gene’ model was postulated. In soybean three out of four QTLs detected for resistance to the soybean cyst nematode were race-specific (Concibido *et al.* 1997). These results indicate that it is likely that minor gene-for-minor gene interactions occur in quantitative resistance. In Chapter 3 the interaction of the two QTLs of *S. chacoense* $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}chc}B$ and eight *M. hapla* populations is described. The race B populations of *M. hapla* showed to be virulent to both resistance genes. Also Janssen *et al.*

(1997a) demonstrated the presence of virulent populations of *M. hapla* towards different *Solanum* species. In their research the tested *M. hapla* were not classified as cytological races A or B. Within race A only one population showed interaction with the $R_{Mh\text{-}chc}B$ gene. This provides a slight indication of the existence of a 'minor gene-for-minor gene' model, although for a concluding proof more research is required. The question of whether the race-non-specific QTL may also be race-specific can only be answered when a larger number of races are tested. If the 'minor gene-for-minor gene' model also refers to the resistance to *M. hapla*, the durability of this resistance might be comparable to the durability of the qualitative resistance to *M. chitwoodi* and *M. fallax*.

Pathotype scheme

The durability of resistance can be extended by the application of different R-gene combinations. Bioassays with a range of different resistant genotypes on the unselected nematode population are required to gain insight in the variation of virulence already present. In this thesis the existence of virulence within the *M. hapla* and *M. chitwoodi* populations is demonstrated with a limited amount of resistant *Solanum* genotypes. Van der Beek (1997) and the results of Chapter 3 show that the populations of *M. hapla* are genetically heterogenic. In addition, the variation within the populations of *M. hapla* makes the genetic analysis of the resistance more difficult.

A pathotype scheme with differential clones, comparable to the international pathotype scheme of the potato cyst nematodes (Kort *et al.* 1977) can help to find new resistance genes in wild species. Such a pathotype scheme describes nematode populations that can or cannot reproduce on a given host genotype. Until now the lack of resistances within the *Solanum* species did not allow classification of pathotypes. The differences between populations that represent specific pathotypes can be very small and difficult to quantify in a bioassay. Therefore, a pathotype scheme should not only be based on arbitrary use of Pf/Pi values. The classification of the pathotypes Pa2 and Pa3 of *G. pallida* is based on the ability to reproduce on the susceptible *S. vernei* hybrid 62.33.3 as indicated by Pf/Pi. This appeared to be independent from the genetic characterization of the *G. pallida* populations (Folkertsma *et al*. 1996a). In contrast to the *G. rostochiensis* populations with a restricted genetic variation, the *G. pallida* populations are more variable and comprise mixtures of virulence gene frequencies. Furthermore the differential clone *S. vernei* hybrid 62.33.3 is not fully resistant to the Pa2 pathotype used in the official trial (pers. com. A.N.O. Rookmaker, Averis Seeds). Therefore, the pathotype scheme of the potato cyst nematodes should be reconsidered. The gene pool similarity concept as proposed by Bakker *et al.* (1993) would be an appropriate tool to distinguish the different nematode populations. The gene pool similarity concept implies that in absence of selection pressure the molecular similarity of the populations will reflect on the virulence loci. This concept was mainly illustrated for the control of potato cyst nematodes, having a poor reproduction capacity and poor dispersal ability. Compared to the *Globodera* spp. the *Meloidogyne* spp. will have a higher reproduction capacity due to more generations during a growing season and the broader host range. Nevertheless this concept can also apply to the *Meloidogyne* spp.. Therefore, molecular markers are needed to state that one pathotype share the same alleles for reproduction on a given plant genotype. Breeders can subsequently use

distinct genetic clusters of the nematode populations as a guide to screen for broad-spectrum resistance. The classification of the two races of *M. chitwoodi* has also been based on solely the Pf/Pi value (Santo & Pinkerton 1985). The results of Chapter 6 show a similar response of the race 2 populations and the virulent race 1 population. Molecular analysis of the *M. chitwoodi* populations might result in a new classification of the races and pathotypes. A proper characterization of the resistance genes and nematode populations will eventually lead to a better knowledge of the amount of virulence factors present already in the nematode populations.

Recent observations in Malta indicate that the root-knot nematode species *M. javanica* is an emerging pest for potato cultivation, causing severe galling on the tuber surface (Vovlas *et al.* 2005). Also the recently described new root-knot nematode species *M. minor,* causes in potato similar symptoms as *M. chitwoodi* and *M. fallax* (Karssen *et al*. 2004). Future research is required to examine the value of the current set of resistance genes against a wider range of Meloidogyne species.

Development of molecular markers

One of the well known benefits of MAS is an early selection in the breeding programme and avoiding expensive and time consuming bioassays like the bioassay for resistance to root-knot nematodes. Furthermore the molecular marker techniques are not influenced by the environment in contrary to bioassays. Because there is no root-knot nematode resistance available in potato genotypes with an acceptable level of agronomical value, the long way of introgression breeding has to be followed. The use of molecular markers offers the breeder a tool of a very early selection without efforts to generate additional material for a bioassay. The profits of MAS are among others depending on the heritability of the trait and its mode of inheritance. We showed in chapter 5 and 6 that the resistance to *M. chitwoodi* and *M. fallax* from the wild species *S. fendleri*¸ *S. hougasii* and *S. bulbocastanum* have a monogenic inheritance. In practical breeding these simple qualitative phenotypes with high heritability benefit only in the sense of assay efficiency. Furthermore the two mentioned nematode species are quarantine organisms, requiring the bioassay only to be performed under strict regulations. Therefore, many of these bioassays could efficiently be replaced by molecular marker techniques. The resistance to *M. hapla* from the wild species *S. chacoense* and *S. tarijense* have a low heritability. The large environmental variation in the bioassay will lead to frequent loss of resistance when MAS is not applied as has been shown in Chapter 2. For the development of potato cultivars with improved resistance to *M. hapla* the potato breeder will benefit much more from molecular markers as a selection tool. Besides these trait by trait benefits, MAS will give the breeder a tool to create combinations of phenotypically similar resistance genes in a variety, so-called pyramiding of resistance genes, as will be discussed later.

Molecular analyses of quantitative resistance

Initial results of bioassays with clones derived from the *M. hapla* resistant wild species *S. chacoense* (Chapter 2) and *S. tarijense* (Chapter 4), demonstrated continuous distribution of resistance scores and a low heritability, suggesting the involvement of QTLs. In contrast to the

study of monogenic characteristics, a genome wide marker coverage is essential to allow QTL studies. Because both wild species are diploid and crossable with diploid *S. tuberosum,* the use of a diploid mapping population was obvious. Especially the construction of a genetic map can be performed with much more ease in a diploid population. Compared with a tetraploid population the construction of a genetic map of a diploid population will take less effort. Bradshaw *et al.* (2004) failed to produce a complete linkage map of a tetraploid mapping population using 38 AFLP primer combinations and 23 SSRs. We produced genetic maps of the *S. chacoense* and *S. tarijense* mapping population using ten AFLP primer combinations. The use of a diploid mapping population is also a major advantage for the integration of breeding and research. In this way the ease of mapping and the more rapid introduction of the genes of interest into cultivated potato in diploid way, are combined. In many QTL studies the association found in the tested mapping population between the markers and a QTL or even a major gene can not always directly be extended to other populations with a different genetic background. This drawback is not expected in the followed approach of a combined genetic QTL study and the introgression of the resistance.

Molecular analyses of qualitative resistance

In order to map the genes involved in the monogenic inherited resistance the construction of a complete genetic map is not necessary. Because the resistance against *M. chitwoodi* and *M. fallax* from the wild species *S. fendleri* (Chapter 5)*, S. hougasii* and *S. bulbocastanum* (Chapter 6) was inherited monogenically, only a partial linkage map of one of the four homologous chromosomes in the vicinity of the resistance gene needed to be constructed. Therefore, the problematic construction of a complete genetic map of a polyploid mapping population could be avoided. With the BSA approach (Michelmore *et al.* 1991) a large amount of AFLP primer combinations could be tested in a short period of time, resulting in a linkage group of 11 AFLP markers in the vicinity of the $R_{Mc1\text{-}fen}$ locus (Chapter 5) and a linkage group of 14 AFLP markers in the vicinity of the $R_{Mc1\text{-}hgs}$ locus (Chapter 6). A disadvantage of constructing only a partial linkage map is the anticipated lack of markers that is homologous (co-migrating to AFLPs with known chromosomal positions) necessary to align the linkage group to linkage groups in reference maps. In Chapter 5 the method of map alignment using co-migrating AFLP markers as suggested by Rouppe van der Voort *et al.* (1997a) indeed failed to align the linkage group. By sequencing the linked AFLP markers and application of the sequence information for the design of primers, we were able to assign the resistance gene to the chromosome number.

Implementation of molecular markers in a potato breeding programme

The implementation of molecular markers in a breeding programme of potato depends on many factors. The costs per data point, the breeding goal and the clonal generation to apply selection criteria play an important role in the decision to utilise molecular markers in a breeding programme. Although most recent molecular studies are performed with molecular markers like AFLP and SSRs, these techniques are not suitable for MAS in a commercial breeding programme. The AFLP markers used in our genetic studies are very efficient in genetic studies but technically demanding and expensive. Therefore, AFLP markers should be converted into a single locus PCR marker, preferably without the necessity of an enzymatic restriction step like

for CAPS markers. MAS should be applicable in both diploid as well as in tetraploid breeding programmes. In order to follow the marker in tetraploid clones, the molecular marker should not give to many bands, preferable only one allele specific amplification product. The markers have to be robust in PCR conditions and low in costs. Recombination between a marker and the gene of interest would nullify the value of the marker. Therefore, markers should be close to the target locus, and all offspring without the marker should be discarded or two markers, on both sides of the target locus, should be used. In an ideal situation the marker is based on the sequence of the gene of interest.
In most potato breeding programmes the selection in the first two years is performed on visually observable characters, such as length of stolons, malformations of the tuber, amount of tubers and tuber size. After one year of field selection, the number of clones is reduced with approximately 90%. Performing MAS after the first field selection will, therefore, drastically reduce the marker genotyping costs. The costs per data point for MAS, however, depend on the volume. Application of MAS either before the development of greenhouse tubers or after the first field selection should be based on the costs per data point versus the costs made for planting, nursing and storage of clones. Besides this high throughput MAS with emphasis on low costs another strategy of implementation of MAS can be perceive: the targeted application of MAS for the development of progenitor lines. To increase the change for offspring with the genes of interest in the latter strategy, the tetraploid progenitor clones should have these genes in at least a duplex way. Because of the relative ease of genetics at the diploid level and the transfer of the majority of the alleles of the diploid parent in the progeny, it is preferred to apply this strategy in diploid crossing parents.

Pyramiding of resistance genes

The application of molecular markers also allows novel and additional goals to be achieved: (1) phenotyping can be replaced by genotyping, and (2) breeding by design (Peleman & Rouppe van der Voort 2003) to generate intended genetic combinations, including the pyramiding of resistance genes (Zhao *et al.* 2005). When phenotyping is replaced by genotyping, combinations of genes can be distinguished from single genes, although the disease or quality assay result in similar phenotypes. As an example black spot bruising could be mentioned where either lack of thyrosine substrate as well as lack of PPO enzyme activity would equally result in bruising resistance. In this Thesis, a number of resistance genes have been identified, that could be used to design a breeding strategy for more durable resistance. This is also referred to as pyramiding of resistance genes. The hypothesis is that a set of resistance genes in one plant would be harder to overcome by the pathogen, or might have resistance to a broader set of virulence genes in the pathogen. To have a more durable resistance due to combinations of resistance genes, these resistance genes should interact in different ways with the pathogen. For example, a resistance gene preventing the pathogen to enter the plant combined with a resistance gene preventing the formation of spores. An example of broader resistance due to pyramiding is the combination of resistance genes acting against different virulence genes of the potato cyst nematode. The present way to determine the resistance against the potato cyst nematodes is performing a bioassay with a population with unknown virulence genes. In that situation the *Gpa2* gene (Rouppe van der Voort *et al.* 1997b) in a

potato clone will prevent the avirulent nematodes in the population to multiply, but because of the low frequency of the avirulent alleles in the tested nematode population this potato clone will be scored as susceptible. Together with other genes the *Gpa2* gene might be the cause of the difference between the Dutch qualification of 'high resistant' and 'resistant'. Pyramiding of genes can be performed with qualitative genes and QTLs. The implementation of QTLs in a breeding program is depending on the amount of explained variance. QTLs with low explained variances, like $R_{Mh\text{-}chc}B$ (Chapter 2), will not easily be used in a high throughput MAS. Also because $R_{Mh\text{-}chc}B$ did not have an effect on all other *M. hapla* populations tested (Chapter 3), suggesting a low frequency of the avirulent alleles of these populations. A breeder would prefer to combine QTLs with a higher explained variance, for instance by pyramiding the genes $R_{Mh\text{-}chc}A$ (Chapter 2) and $R_{Mh\text{-}tar}$ (Chapter 4). The resistance genes $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}tar}$ alone show an insufficient level of resistance. A combination of these two genes might have a broader resistance. A prerequisite is that the two mentioned resistance genes are acting against different virulence genes, which has to be confirmed by future research.

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Summary & Samenvatting

The development of potato varieties with resistance towards the potato cyst nematode, allowed a dramatic decrease of the use of nematicides. Subsequently the population of the free living nematodes and the root-knot nematodes (*Meloidogyne* spp.) has increased. Among the root-knot nematodes, three *Meloidogyne* species are important in the potato cultivation in The Netherlands: *M. hapla*, *M. chitwoodi* and *M. fallax*. The latter two species are the most harmful because they cause malformation in the tubers and are quarantine organisms. Until now no resistant potato cultivars are available. Several wild species of potato show resistance, but several back crossings are required to develop a root-knot nematode resistant potato cultivar. Selection based on molecular markers will speed up this development and will replace the expensive and time consuming bioassays, which have to be carried out under strict quarantine conditions. In this thesis diploid as well as polyploid wild *Solanum* species are used for the introgression of resistance genes in cultivated potato, and to analyse the inheritance of the various resistances.

Chapter 2 describes the genetic analysis of the diploid wild species *Solanum chacoense* with resistance against *M. hapla* and *M. fallax.* An AFLP based linkage map was constructed, resulting in twelve linkage groups of either parent of the mapping population with a total length of 685 and 596 cM each. Due to the large differences in AFLP patterns between *S. chacoense* and cultivated potato, chromosome numbers could not be assigned to the linkage groups containing the QTLs. Two QTLs for resistance against the *M. hapla* population Hi were identified in this population. These two QTLs, $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}chc}B$, explained 38% and 13% of the total phenotypic variance of the resistance to *M. hapla*. As a consequence of the large variation in the results of the bioassay, phenotypic selection may lead to the loss of these resistance QTLs. This loss can be prevented by marker assisted selection with the markers flanking the QTLs. The genetic analysis of the resistance against *M. fallax* resulted in the detection of one QTL explaining 14% of the phenotypic variance. The high level of resistance to *M. fallax* as observed in the resistant parent was not observed in the progeny. In view of the modest results that can be expected from *S. chacoense*, we cannot recommend breeding efforts with this germplasm. Better results in genetic analyses and introgression of resistance against *M. fallax* were achieved in the mapping studies with the wild *Solanum* species *S. fendleri* (Chapter 5), *S. hougasii* and *S. bulbocastanum* (Chapter 6).

The differential interaction of eight *M. hapla* populations to the resistance QTLs, $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}chc}B$ was studied in Chapter 3. The $R_{Mh\text{-}chc}A$ allele allows recognition on virulence factors present in all six tested cytological race A populations. Although the allele $R_{Mh\text{-}chc}B$ also showed a reduction in the amount of formed egg masses of population Ham, this allele had only an additive effect with population Hi, the population initially used for the QTL analysis (Chapter 2). No effect of both alleles was noticed for the two race B populations. Besides the distinctive difference in reproduction between race A and B, both races can now also be divided based on their differences in virulence to the $R_{Mh\text{-}chc}A$ allele. Furthermore, the molecular fingerprints allow too clearly distinguish between the races A and B. Because the resistant parent of the mapping population is susceptible to the race B populations, it is not worthwhile to check the used mapping population for other resistance QTLs. The breeder should therefore search for new sources of resistance to the race B populations in the wild species. Looking at the higher level

of reproduction, the population Han would be the most appropriate population to be used in that survey.

In Chapter 4 the diploid wild species *S. tarijense* was used as the source of resistance to *M. hapla.* The non absolute level of resistance and the continuous distribution of produced egg masses in the segregating offspring, suggests that the resistance is under polygenic control. Therefore a linkage map of the BC1 mapping population was developed as prerequisite of QTL analysis. The assignment of a chromosome number to the linkage groups was simple, because a detailed genetic map was already constructed of the susceptible parent. The linkage groups of the resistant female parent were subsequently aligned to the genetic map of the susceptible male parent using the AFLP markers that were heterozygous in both parents (so-called ab×ab markers). The QTL mapping approach resulted in only one locus, $R_{Mh\text{-}tar}$, on chromosome 7. In contrast to QTL analysis that leads to a broad QTL interval, qualification of the resistance will lead to a more precise map position of the resistance locus. For that case the progeny of the mapping population was arbitrary classified in two groups: 1) a resistant group with the amount of less than1 egg mass and 2) a susceptible group with more than 10 egg masses. Via this approach the $R_{Mh\text{-}tar}$ resistance gene could be located on a distal position of the short arm of chromosome 7, and the low amount of singletons offered an indirect validation of the classification of the resistance phenotypes in discrete groups. The value of the $R_{Mh\text{-}tar}$ gene for the potato breeding is depending on the allele frequency of the associated virulence genes in the *M. hapla* populations and the geographic distribution of such populations.

In contrast to the research with *M. hapla* as described in Chapter 2 and 4, the progeny of the mapping populations with resistance against *M. chitwoodi* and *M. fallax* (Chapter 5 and 6) could be easily classified as resistant or susceptible. This suggests that resistance is mediated by a single R-gene. The resistance against both nematode species, *M. chitwoodi* and *M. fallax*, was absolutely correlated in all three mapping populations, derived from *S. fendleri* (Chapter 5), *S. hougasii* and *S. bulbocastanum* (Chapter 6). In Chapter 5 the monogenic resistance against *M. chitwoodi* and *M. fallax* was mapped in an inter-specific and an intra-specific mapping population of the tetraploid wild species *S. fendleri*. By using the so-called Bulked Segregant Analysis (BSA) method, a large amount of closely linked markers was identified efficiently. In the intra-specific mapping population a remarkably lower amount of linked AFLP markers was found. This can be due to the fact that *S. fendleri* is a self-fertilising species, showing a lower level of genetic polymorphisms. On the other hand the genetic dissimilarity between the wild species introgression segments and the recurrent potato parents may have caused an elevated level of polymorphic markers in the BC_2 inter-specific mapping population. Besides the larger amount of polymorphic markers, the utilisation of inter-specific mapping populations is an advantage for the breeder, because back-crossings are already made, necessary to introgress the resistance into potato. Based on the sequence of an $R_{Mc1\text{-}fen}$ locus linked AFLP marker, a PCR marker was developed. This marker was used to map its position in a reference map, were it was located on the distal position of the long arm of chromosome 11. The chromosomal position of the $R_{Mc1\text{-}fen}$ locus was confirmed by using the chromosome 11 specific CAPS marker M39b. The $R_{Mc1\text{-}fen}$ gene is located in a cluster of resistance genes, containing the highly related *Rmc1* gene and other fungus, virus and nematode resistance genes. This PCR marker,

however, can not yet be used in marker assisted selection because 40% of the progeny with the PCR product will not contain the resistance allele.

Chapter six describes the localisation of resistance against *M. chitwoodi* and *M. fallax* in a BC2 population of the hexaploid wild species *S. hougasii*. Also this study used the BSA method, resulting in a linkage group of 14 AFLP markers and the monogenic resistance locus $R_{Mc1\text{-}hou}$. Similar to the linkage group of *S. fendleri,* the linkage group of *S. hougasii* has been positioned on the distal position of the long arm of chromosome 11. The differential interaction between the resistance genes $R_{Mc1\text{-}blb}$, $R_{Mc1\text{-}fen}$ and $R_{Mc1\text{-}hou}$ with six *M. chitwoodi* populations has been studied. Differences in virulence were not found between race 1 and race 2, but, remarkably, within the two races. Populations Ccl and Cbh seem to have a similar virulence spectrum, while these populations are described as respectively race 1 and race 2. The presence of virulence in the populations will have a negative effect on the durability of the resistance. Because the three resistance genes against *M. chitwoodi* and *M. fallax* from the wild species *S. bulbocastanum*, *S. fendleri* and *S. hougasii* are mapped on the same locus and act against the same spectrum of virulence genes, no additional value is expect from the strategy of pyramiding these genes. Consequently, the breeder has to search for new resistance sources against the virulent nematode population Ccl and/or Cbh, in order to develop a potato with broad spectrum resistance.

The resistance genes described in this thesis can be introgressed in potato with the help of molecular markers. Moreover the presence of individual resistance genes can be monitored by molecular markers, which is a prerequisite for pyramiding of R-genes. Pyramiding of the resistance genes may lead to a resistance with a broader spectrum, and/or may offer a more durable resistance, providing that it can be performed with loci with a complementary resistance spectrum.

Samenvatting

Door de ontwikkeling van aardappelmoeheid resistente aardappelrassen is het gebruik van nematiciden drastisch afgenomen, met als gevolg een opkomst van de vrijlevende nematoden en het wortelknobbelaaltje, *Meloidogyne* spp.. In Nederland komen drie voor de aardappelteelt belangrijke *Meloidogyne* soorten voor, *M. hapla*, *M. chitwoodi* en *M. fallax* waarvan de laatste twee het meest schadelijk zijn in de aardappelteelt en bovendien een quarantaine status hebben. Tot nu toe is in de huidige aardappelrassen geen resistentie tegen deze nematoden aanwezig. Verschillende wilde soorten zijn echter wel resistent, maar het vergt nog vele terugkruisingen voordat een wortelknobbelaaltje resistente aardappelras op de markt kan verschijnen. Selectie op basis van moleculaire DNA merkers zal de ontwikkeling van resistente aardappelrassen versnellen en bovendien vervangt deze moleculaire merker analyse de dure resistentietoets die onder quarantaine omstandigheden plaats moet vinden. In deze thesis zijn zowel diploïde als polyploïde wilde soorten gebruikt om resistente aardappelen te ontwikkelen en de resistentie te karteren.

Hoofdstuk 2 beschrijft de genetische analyse van de diploïde wilde soort *Soalnum chacoense* met resistentie tegen *M. hapla* en *M. fallax*. Er werden koppelingskaarten met AFLP merkers gemaakt van beide kruisingsouders met als resultaat twee genetische kaarten van 12 koppelingsgroepen met een totale lengte van 685 en 596 cM. Door de grote genetische verschillen tussen deze wilde soort en de cultuuraardappel was het niet mogelijk om op basis van AFLP techniek de chromosoomnummers toe te wijzen aan alle koppelingsgroepen. Er werden twee QTLs gevonden voor resistentie tegen de *M. hapla* populatie Hi. Deze twee QTL's, $R_{Mh\text{-}chc}A$ en $R_{Mh\text{-}chc}B$, hadden een verklaarde variantie van respectievelijk 38% en 13%. De grote variatie in de resistentietoets is de reden dat fenotypische selectie kan leiden tot verlies van de afzonderlijke resistentiegenen. Dit kan voorkomen worden door de fenotypische selectie te vervangen door merker gestuurde veredeling op basis van de gevonden flankererende merkers. De genetische analyse van de resistentie tegen *M. fallax* resulteerde in de detectie van één QTL met slechts 14% verklaarde variantie. Bovendien werd de mate van resistentie tegen *M. fallax* van de resistente ouder niet terug gevonden in de nakomelingen. Betere vererving van de resistentie tegen *M. fallax* werd verkregen in de mappingpopulaties met resistenties uit *S. fendleri* (Hoofdstuk 5), *S. hougasii* en *S. bulbocastanum* (Hoofdstuk 6).

De differentiele interactie tussen de QTL's $R_{Mh\text{-}chc}A$ en $R_{Mh\text{-}chc}B$ en acht *M. hapla* populaties werd bestudeerd in Hoofdstuk 3. Het $R_{Mh\text{-}chc}A$ allel herkent de virulentie factoren van alle getoetste cytologische race A populaties. Alhoewel het $R_{Mh\text{-}chc}B$ allel ook een reductie in het aantal gevormde eiproppen laat zien bij de populatie Ham, had dit allel alleen een significant additioneel effect bij populatie Hi, de *M. hapla* populatie die in Hoofdstuk 2 gebruikt werd voor de QTL analyse. Beide QTL's hadden geen effect op de race B populaties van *M. hapla*. Naast de het verschil in meiose tussen race A en race B, kunnen de twee race's dus nu ook onderscheiden worden op basis van de virulentie tegen het $R_{Mh\text{-}chc}A$ allel. Ook met moleculaire merkers is een duidelijk onderscheid te maken tussen race A en race B. Omdat de resistente ouder ook vatbaar is voor de race B populaties heeft het geen zin om de mapping populatie te onderzoeken op aanwezigheid van resistentie QTL's. De kweker zal dus op zoek moeten gaan naar nieuwe bronnen van resistentie tegen race B. Gezien de mate van reproductie van

populatie Han, is dit de meest aangewezen populatie om nieuwe bronnen van resistentie op te sporen.

In Hoofdstuk 4 is de diploide wilde soort *S. tarijense* gebruikt als resistentie bron voor *M. hapla*. Het niet absolute niveau van resistentie en de continue verdeling van de geproduceerde eiproppen in de nakomelingen, suggereert dat de resistentie in deze wilde soort polygeen is. Daarom werd van de BC1 karterings populatie een volledige genetische kaart, nodig voor QTL analyse, gemaakt. Het toekennen van de chromosoomnummers aan de koppelingsgroepen was in deze populatie eenvoudig omdat van de vatbare ouder al een zeer gedetaileerde genetische kaart bestond. De koppelingsgroepen van de resistente moeder kon vervolgens met ab×ab merkers gekoppeld worden aan de genetische kaart van de vatbare vader. De QTL analyse resulteerde in de detectie van slechts één QTL, $R_{Mh\text{-}tar}$, op chromosoom 7. De resistentie werde ook kwalitatief gekarteerd om zodoende een nauwkeurige locatie van het resistentiegen te krijgne. De nakomelingen van de onderzoekspopulatie werden daartoe in twee groepen verdeeld op basis van een arbitraire indeling van 0-1 eiprop voor de resistente groep en meer dan 10 eiproppen voor de vatbare groep. het $R_{Mh\text{-}tar}$ resistentie gen werd gekarteerd distaal op de korte arm van chromosoom 7. De waarde voor de aardappelveredeling van het $R_{Mh\text{-}tar}$ gen hangt af van de allelfrequentie van de bijbehorende virulentiegen in de *M. hapla* populaties en de geografische verdeling van deze populaties.

In tegenstelling tot het onderzoek met *M. hapla*, beschreven in Hoofdstukken 2 en 4, kunnen de nakomelingen van onderzoekspopulaties met resistentie tegen *M. chitwoodi* en *M. fallax*, beschreven in de hoofdstukken 5 en 6, eenvoudig ingedeeld worden in een resistente en vatbare groep. De resistentie tegen *M. chitwoodi* en *M. fallax* was in alle drie onderzoekspopulaties, *S. fendleri* (Hoofdstuk 5), *S. hougasii* en *S. bulbocastanum* (Hoofdstuk 6), volledig gecorreleerd. In Hoofdstuk 5 is in de tetraploide wilde soort *S. fendleri* de monogene resistentie tegen *M. chitwoodi* en *M. fallax* gekarteerd in een interspecifieke en een intraspecifieke onderzoekspopulatie. Daarbij werd gebruik gemaakt van de Bulked Segregant Analysis (BSA) methode, waardoor zeer efficiënt een groot aantal gekoppelde merkers gevonden werd. In de intraspecifieke karteringspopulatie werd een opvallend lager hoeveelheid gekoppelde AFLP merkers gevonden, hetgeen verklaard kan worden doordat *S. fendleri* een zelfbevruchtend soort is en daardoor genetisch minder polymorfie bezit. Bovendien kunnen de genetische verschillen tussen de wilde *S. fendleri* en de cultuuraardappel een oorzaak zijn van de hogere mate van polymorfie in de interspecifieke onderzoekspopulatie. Naast de hogere hoeveelheid merkers is het voor de kweker vooral voordelig om interspecifieke populaties te gebruiken, omdat dan al een begin wordt gemaakt met de vele terugkruisingen die nodig zijn om de resistentie in een kwalitatief hoogwaardig aardappelras te krijgen. Op basis van de sequentie van een aan het $R_{Mc1\text{-}fen}$ locus gekoppelde AFLP merker, werd een PCR merker ontwikkeld. Deze PCR merker werd gebruikt in een referentie populatie waar het gekarteerd werd op de distale positie van de lange arm van chromosoom 11. De locatie van de koppelingsgroep met het $R_{Mc1\text{-}fen}$ gen werd bevestigd door de kartering van het chromosoom 11 specifieke CAPS merker M39b. Het $R_{Mc1\text{-}fen}$ gen ligt in een cluster van resistentiegenen, waaronder de sterk gerelateerde *Rmc1* gen en andere schimmel, virus en nematoden resistentiegenen. De PCR merker kan echter nog niet gebruikt worden voor merker gestuurde

veredeling, omdat in 40% van de nakomelingen met het PCR product het resistentie allel afwezig is.

In Hoofdstuk 6 is de resistentie in de hexaploide wilde soort *S. hougasii* in een BC2 populatie gekarteerd. Ook hier werd de BSA methode gebruikt en dit resulteerde in een koppelingsgroep met 14 AFLP merkers en de monogene resistentie locus $R_{Mc1\text{-}hou}$. Deze koppelingsgroep werd net als de koppelingsgroep van *S. fendleri* gekarteerd op de distale positie van de lange arm van chromosoom 11. Vervolgens werd de differentiele interactie tussen de resistentiegenen $R_{Mc1\text{-}blb}$, $R_{Mc1\text{-}fen}$ en $R_{Mc1\text{-}hou}$ met zes *M. chitwoodi* populaties bestudeerd. Opmerkelijk was dat de virulentie verschillen niet gevonden werd tussen race 1 en race 2, maar binnen de race's. Voorlopig lijken de populaties Ccl en Cbh een gelijke virulentie spectrum te hebben, terwijl deze populaties beschreven staan als respectievelijk race 1 en race 2. De aanwezigheid van virulentie in de populaties zal de duurzaamheid van de huidige resistentie negatief beïnvloeden. Omdat de drie gekarteerde resistentiegenen tegen *M. chitwoodi* en *M. fallax* uit de wilde soorten *S. bulbocastanum*, *S. fendleri* en *S. hougasii* niet alleen op dezelfde locus liggen, maar bovendien hetzelfde werkingsspectrum hebben, wordt weinig additionele waarde verwacht van de strategie van stapelen van deze resistentie genen. De kweker zal dus op zoek moeten gaan naar nieuwe resistentiebronnen tegen de virulente nematoden populaties Ccl en/of Cbh, om een breed werkend resistente aardappel te ontwikkelen.

De in deze thesis gekarteerde resistentiegenen kunnen met behulp van moleculair merkers efficiënt in aardappel ingekruist worden. Bovendien zijn de resistentiegenen door de moleculaire merkers onderscheidbaar en daardoor wordt het mogelijk om resistentiegenen gericht te combineren. Dit zogenaamde stapelen van resistentiegenen zou kunnen leiden tot een bredere resistentie of een duurzamer resistentie.

Eén van de meest gebruikte woorden in het nawoord van proefschriften is “eindelijk”. Als één proefschrift in aanmerking komt voor het gebruik van dit woord, dan is het dit proefschrift wel. Iets meer dan vier jaar na het beëindigen van het praktische werk is het **eindelijk** zover. Ik kan mijn promotieonderzoek afronden met dit proefschrift en de verdediging ervan. Of zoals veel vrienden en familie het noemen: mijn “studie” afronden.

Traditiegetrouw wordt in het nawoord iedereen bij name genoemd die een rol in het promotieonderzoek heeft gespeeld. Voor de goede orde: daar hoor jij ook bij, al wordt je niet bij name genoemd.

Eén van degene die ik wel bij name wil noemen is Jan van Loon, omdat hij mij aanspoorde dit onderzoek uit te voeren. Gezamenlijk met Stet Holland en KARNA vormde Hettema de Stichting Bevordering Veredelingsonderzoek (SBV) die mijn onderzoek financierde. In vier jaar tijd kan veel gebeuren, zo bestaat de huidige SBV na overname, fusie en naamswijziging nu uit HZPC en Averis Seeds. Van de SBV wil ik oud- en nieuwgedienden: Frits, Paul, Jacob, Nico, Robert, Martin, Peter en Nick bedanken voor de prettige samenwerking.

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Tot slot wil ik het thuisfront bedanken. Ingrid, jij hebt altijd achter de beslissing gestaan om te promoveren. Ook in de moeilijkere perioden bleef je achter en naast me staan en daarvoor wil ik je enorm bedanken. Nu deze fase is afgesloten hoop ik dat er een rustiger tijd aanbreekt voor ons gezinnetje. Hoe de toekomst er ook ziet, ik zie het met jou, Chris en Anniek met vertrouwen tegemoet.

Zo, …

Nou is't klaar!

Jan Draaistra werd geboren op 16 januari 1970 te Harkema. In 1988 werd het HAVO diploma behaald aan het Chr. Lyceum 'Oostergo' te Dokkum, waarna hij in 1993 de Agrarische Hogeschool Friesland te Leeuwarden succesvol afrondde. De goede resultaten in het laatste jaar werden beloond met de Boelstra-Olivier prijs. Hierna zette hij zijn studie voort aan de Landbouw Universiteit in Wageningen (LUW). Zijn afstudeeronderzoeken heeft hij uitgevoerd bij de vakgroep Nematologie en de vakgroep Erfelijkheidsleer. In 1996 studeerde hij af als plantenveredelaar. In april 1997 is hij in dienst getreden bij Hettema BV te Emmeloord en heeft vanaf 1 november 1997, gedetacheerd in Wageningen, voor de Stichting Bevordering Veredelingsonderzoek (SBV) zijn promotieonderzoek uitgevoerd. Hiervan staan de resultaten beschreven in dit proefschrift. Sinds 1 november 2001 is hij als Manager Breeding werkzaam bij Averis Seeds B.V. te Valthermond.