Genetic Mapping and Pyramiding of Resistance Genes in Potato

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Voor Paps

‘Freedom‘s just another word for nothing left to lose’

Kris Kristofferson

Contents

Chapter 1

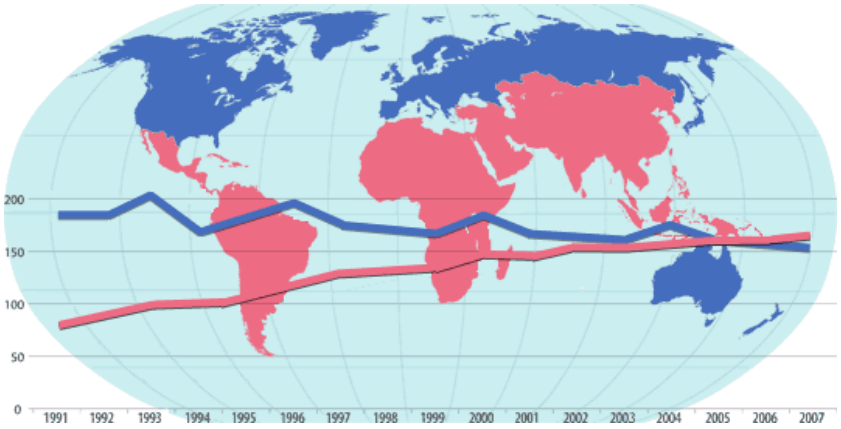
General Introduction

Potato

Potato (*Solanum tuberosum* ssp. *tuberosum*) belongs to the *Solanaceae* family together with other domesticated species such as tomato, capsicum, eggplant, tobacco and petunia. Bittersweet (*S. dulcamara*) and black nightshade (*S. nigrum*) are common *Solanum* species belonging to the European flora. Potato has a variable ploidy level ranging from 2x to 6x where the cultivated potato is an auto-tetraploid (4x). The origin of potato cultivation is situated in the area of lake Titicaca, in the Andes region of South America. There, the domestication of wild potato plants first started. Soon after the invasion of Peru by Spanish conquistadores in the 1500's, the potato was introduced to Europe (Salaman 1949 and http://www.potato2008.org/en). Potatoes were first cultivated in 1573 in Spain, were grown in London by 1597 and reached France and the Netherlands soon after.

Nowadays, according to FAOSTAT, potato production in 2007 reached 320 million tons worldwide. This implies a 4th place in the world food production after wheat, corn and rice. With an increasing production growth since the 1990's the production in Asia, Africa and Latin America exceeds the production of potatoes in the developed countries (Figure1). China is at the moment the leading potato producer of the world. The Netherlands occupies the 9th place with a harvest of 7.2 million tons in 2007. Approximately 155,000 hectare (about 25 %) of the arable land in the Netherlands is planted with potatoes. Seed potato and starch potato represents 20 and 30% of the yield, respectively. The remaining 50% is for consumption, including ware potato, French fries and crisps in The Netherlands (20%) and abroad (30%). The Dutch consume on average 90 kg of potatoes per capita per year (http://faostat.fao.org/ and http://www.potato2008.org/en).

World potato production, 1991-2007



	1991	1993	1995	1997	1999	2001	2003	2005	2007
Countries	million tonnes								
Developed	183.13	199.31	177.47	174.63	165.93	166.94	160.97	159.99	155.56
Developing	84.86	101.95	108.50	128.72	135.15	145.92	152.11	160.12	165.15
WORLD	257.25	301.27	285.97	303.36	301.08	312.86	313.09	320.11	320.71

Figure 1: World potato production
(source: http://www.potato2008.org/en/world/index.html).

The increasing popularity of potato globally is not surprising. With high productions per hectare, a high vitamin C content and high quality proteins, it makes an excellent staple food.

Diseases

Numerous pathogens can infect potato, resulting into more than a hundred different diseases. These diseases are caused by bacteria, fungi, oomycetes, viruses and nematodes. Fortunately most of the diseases have only local importance, but a few diseases make a global impact. Late blight and nematode diseases are most dominant. Late blight, caused by the oomycete *Phytophthora infestans* (Mont.) de Bary, is still the number one threat to potato production. In Latin, *Phytophthora* even means plant destroyer. It is the classic example of how devastating a crop disease can be. In 1845 it was introduced in Belgium by a shipment of seed potatoes from America. The disease spread quickly and in 1845-1847, during the Late Blight epidemics, over one

million people died in Ireland and over 2 million Irish migrated, in a period that has become known as The Irish Potato Famine. The devastation by late blight was exacerbated by the monoculture of potato in Ireland. To make matters worse for us now, in the late 1970's, a new mating type (A2) appeared that was probably introduced in Europe by a shipment of potatoes from Mexico (Fry *et al.* 1993). As a consequence *P. infestans* can now reproduce sexually as well. As a result of meiotic recombination between genotypes with different virulence factors this significantly contributes to the ability to overcome individual resistance genes and combinations thereof.

The consequences of the disease on yield loss are difficult to estimate. The International Potato Centre (CIP) estimated damage in developing countries due to late blight at an average production loss of 15%. This translates into a total production loss of approximately $2.75 billion assuming a price per kg common to developing countries (Anonymous, 1997). In the Netherlands the costs of controlling late blight is estimated at 115.5 million euro's (Haverkort *et al.* 2008).

The dispersal of *Phytophthora infestans* is mainly done by wind. Managing the threat of late blight involves prevention of early infection sources by means of sanitation, monitoring disease outbreak and early warning systems. It is of utmost importance that an early infection is treated with fungicides as cymoxanil and metalaxyl. In organic farming no pesticides are allowed. To lower the risk of yield losses, one strategy is to use early maturing potato plants. In the Netherlands, when infection eventually occurs, the late blight infected foliage must be burned before 1000 leaves per 20 m^2 are infected. Late blight has developed resistance against certain chemicals, such as metalaxyl (Davidse *et al.* 1983). The environmental impact of fungicides is mainly on groundwater, but the overall toxicity is much lower than of herbicides and insecticides. The aim of the "Meerjarenplan Gewasbescherming" was to reduce fungicide use in The Netherlands with 36 % over a 10-year period between 1990 and 2000. However, potato cultivation still relies heavily on application of fungicides, also because of an increased aggressiveness of late blight. The aim of the "Meerjarenplan Gewasbescherming" was therefore not met; fungicide use in The Netherlands had even increased slightly (http://www.gewasbescherming.nl/evaluatie.pdf).

The second important potato disease is caused by nematodes. Most important in Europe are two species of potato cyst nematodes (PCN) *Globodera rostochiensis* and *G. pallida*. Potato cyst nematodes are estimated to cause yield losses of up to 10 % worldwide (Oerke *et al.* 1994). Other nematode species that can infect potatoes are the root-knot nematodes (RKN) (*Meloidogyne* spp.), the lesion nematodes (*Pratylenchus* spp.), potato rot nematode (*Ditylenchus destructor*), sting nematode (*Belonolaimus longicaudatus*) and the stubby-root nematodes (*Paratrichodorus* spp. and *Trichodorus* spp.). Management of nematode pests involves mainly crop rotation, use of nematicides and resistant potato species. Nearly all nematicides are acutely toxic for wildlife and to man. Chemical control of nematodes in the Netherlands has been drastically reduced because of government legislation in reducing soil disinfectants. Due to the "Meerjarenplan Gewasbescherming" and the "Regulering Grondontsmettingsmiddelen", the use of soil sanitation disinfectants was reduced with 88% (http://www.gewasbescherming.nl/evaluatie.pdf)

R-gene mediated resistance

Disease resistance genes (*R*-genes) play a role in the defence of a plant against pathogens (Dangl and Jones 2001). The plant-pathogen interaction is governed by the pathogens *Avr*-genes and the corresponding plant *R*-genes. This model of interaction was termed the gene-for-gene model by Flor (Flor 1971).When the elicitor coded by the *Avr*-gene and the protein encoded by the *R*-gene recognise each other and interact, a defence process will be initiated. In *R*-gene mediated defence against late blight as well as against nematodes the interaction will be followed by signal transduction pathways that lead to a hypersensitive response (HR). The hypersensitive response leads to cell death which includes the death of the pathogen, and might be observed as a small localised necrotic spot that develops in the plant. An effective HR will obstruct growth of *P. infestans* and thereby blocks further infection of neighbouring cells and ultimately the rest of the plant. In nematode–plant interactions a successful HR has a similar effect, and the development of the feeding cell on which the nematode feeds will not occur or will be stopped, causing the nematode to starve and die.

History of deployment of *R*-genes in potato

The value of genetic resistance as mediated by *R*-genes was recognised long before its characterisation by Flor (1971). Breeders have successfully deployed *R*-genes for almost any pathogen in almost any crop species, starting from the first half of the 20th century. Various attempts have been made to introduce late blight and nematode resistance into potato, where some examples are more successful than others. The first disease resistance purposefully introduced in cultivars was probably against the fungus *Synchitrium endobioticum*, the causal agent of wart disease. Also originating from the Andes, this fungus emerged in Europe in the late 19th century. First in the UK where it was found in 1876, from where it spread to the rest of Europe, arriving in The Netherlands in 1915 (Baayen *et al.* 2006). Within the existing breeding pool of cultivars a Mendelian factor was discovered that was monogenically transmitted and was sufficient for resistance against wart disease (Langerfeld *et al.* 1994). This led to disease management strategies which included prohibition of susceptible cultivars. In the 1940's however, wart's appeared on formerly resistant cultivars, demonstrating that new pathotypes are circulating in Europe, and prompted the need to incorporate additional resistance genes. In due course the allele frequency of resistance to wart disease race 1 has reached such a high level, that many of the currently released cultivars have this resistance (even unintended).

When late blight became a problem in the middle of the 19th century, different solutions were proposed. The first solution was a chemical one: Bordeaux mixture, a mixture of lime with a solution of copper sulphate. The alternative solution was conceived in the 1900's, after R.N. Salaman had obtained a few tubers from a wild potato species from Kew botanical gardens in England, in which disease resistance against *P. infestans* was discovered. The wild potato species later turned out to be *S. demissum* and proved almost, but not completely resistant to infection (Salaman 1910). Eleven *R*-genes (*R1*-*R11*) introgressed from *S. demissum* were later identified (Black *et al.* 1953; Eide *et al.* 1959; Malcolmson 1969; Malcolmson and Black 1966), and were deployed in potato cultivars reaching the market in the 1950's and 1960's. However, unfortunately enough, soon after the introduction of these resistant potato cultivars the pathogen demonstrated its ability to change virulence patterns, resulting in new races that could overcome the *S. demissum* derived *R*-genes. This was a great disappointment and the trust in *R*-genes was lost. For a long time, breeding efforts for

R-gene resistance against *P. infestans* was at a stand still. In addition, cheap and effective synthetic pesticides became available, nullifying the incentive for resistance breeding. Alternative breeding strategies were conceived, concentrated on breeding for the so-called “field resistance” or “horizontal resistance”, a resistance not mediated by *R*-genes (Colon *et al*. 1995; Landeo *et al*. 1995; Turkensteen 1993). While this strategy has been successful in several crop – pathogen interactions, this strategy did not provide any improvement in late blight resistance, unless it was associated with an undesirably late maturity. Increased environmental awareness of the public has recognised the harmful effects of pesticides. After an intermission of about 40 years, the focus in breeding is back on *R*-gene mediated resistance to control late blight (Allefs *et al.* 2005). In 2006 and 2007 two new potato cultivars, Toluca and Bionica, entered the market containing the late blight resistance gene *Rpi-blb2* originating from *S. bulbocastanum* (rassenlijst).

Early work on resistance breeding against nematodes was concentrated on the yellow potato cyst nematode *G. rostochiensis*. Breeders succeeded in producing potato cultivars resistant to *G. rostochiensis*, predominantly by incorporating the *H1* gene from *S. tuberosum* ssp. *andigena* CPC 1673 (Ellenby 1952; Huijsman 1957; Ross 1979). Released in 1966, Maris Piper was the first of a long row of varieties with the *H1* gene. Nowadays, the allele frequency of *H1* is so high that *H1* resistance gene is present in almost all cultivars. In contrast to the *S. demissum R*-genes against late blight, the *H1* resistance has proven to be durable up till now

Breeding for resistance against *G. pallida* was more difficult because of the quantitative inheritance (initially perceived as polygenic) of *G. pallida* resistance. Large effect QTL have been introgressed from wild potato species such as *S. andigena* and *S. vernei*. Much later molecular marker techniques allowed the identification of the genes involved. The resistance genes *H3*, *Grp1* and *Gpa5* for example have been identified as the major sources of *G. pallida* resistance in the current potato gene pool. *H3* originates from *S. andigenum* CPC2802 and was mapped in clone 12601ab1 (Bradshaw *et al.* 1998). *Gpa5* probably originates from *S. vernei* CPC2487 / 2488 and was mapped in dihaploid 3704-76 obtained from the tetraploidy progenitor clone AM 78-3704, a tetraploid hybrid of *S. tuberosum* and several wild species. *Grp1* might also originate from *S. vernei* and was mapped in dihaploid 3778-16 obtained from the tetraploid progenitor clone AM 78-3778. These

progenitors have resulted in approximately 20 (using AM 78-3704) and 5 *G. pallida* resistant cultivars (using AM 78-3778). The *H3* gene is deployed in twelve British and Irish cultivars. On the other hand the *G. pallida* resistant progenitor clone VTN 62-33-3 derived from *S. vernei* CPC2487 / 2488 is ancestral to more than one hundred cultivars. This brief overview on deployment of resistance genes shows that the few genes that have been characterised genetically and tagged with DNA markers are represented in a minority of the resistant cultivars, whereas the vast majority of the *G. pallida* resistant cultivars represent germplasm (i.e. VTN 62-33-3) of which the underlying genes have not been analysed.

The prevalence of the various nematode species depends on disease management systems. The application of nematicides reduced soil infection for all soil organisms equally, but the widespread application of the *H1* gene has caused a shift from *G. rostochiensis* to *G. pallida*. Likewise the introduction of resistance against *G. pallida* will most probably cause a shift from PCN to RKN.

The potato pedigree database (http://www.plantbreeding.wur.nl/potatopedigree) is a very useful resource to provide insight in well used and under exploited sources of resistance. Once identified as a valuable source of resistance, potato breeders have widely used resistant material. The prominent use of wild species is also illustrated by the study of Love (1999), where of 44 prominent north American cultivars, 34 (77%) had an exotic background.

Breeding at the diploid level

The widespread use of valuable resistance genes as described in the previous paragraph is limited to a few widely used sources and comprises half a century of breeding activity. The few sources and the long time requirement are explained by the difficulty to remove undesirable traits from wild donor species. Introgression breeding for resistance can be accelerated by two techniques: pre-breeding at the diploid level and the use of DNA markers. Furthermore, these two tools are interconnected: at the diploid level the power of DNA markers can be fully exploited, in contrast to the tetraploid level, where linkage in repulsion cannot be detected (Li *et al.* 1998).

Breeding at the diploid level requires efficient methods of ploidy manipulation via unreduced gametes and prickle pollinations. Breeding at the diploid level has a number of advantages (Hutten 1994). The most obvious advantage is the crossability of diploid breeding lines with diploid sources of resistance, which cannot be crossed with tetraploids directly. Another advantage is the more efficient selection against undesirable traits at the diploid level. Finally, at the diploid level the power of genetic analysis can be used in its full potential and linkage drag can be removed by searching specific recombination events flanking the *R*-genes.

Pyramiding resistance genes

It is evident that resistance genes are an important tool in the battle against plant diseases and an alternative for pesticide use. A lot of resistance genes are already being used in agriculture and an increasing number are being identified. But there are a couple of drawbacks in resistance gene use. Firstly: most resistance genes give partial levels of resistance, not absolute immunity. And secondly, resistances from *R*-genes can break down. The lack of durability of the *S. demissum* late blight *R*-genes was quite a disillusionment.

Pyramiding (major) *R*-genes can be a solution to improve on both the level of resistance and on durability (Nelson 1972). Also a broader spectrum of resistance can be achieved. Pyramiding is the accumulation of (*R*)-genes into a single genotype or cultivar and can be done using major *R*-genes, defeated *R*-genes, different alleles of one gene, or the same alleles (allele-dosage).

A rather limited number of studies have reported on pyramiding of *R*-genes in different plant-pathosystems. The reason probably being that genotyping the pyramiding population needs reliable molecular markers that are not always at hand. The studies that do exist have varying outcomes. Most studies showed a higher level of resistance. Barloy *et al*. (2007) showed an improvement in resistance against cereal cyst nematodes in wheat when pyramiding resistance genes *CreX* and *CreY*. Several groups have reported on the pyramiding of bacterial blight resistance genes in rice and observed higher resistance levels and obtained additionally, a broader spectrum of resistance (Huang *et al.* 1997; Singh *et al.* 2001; Yoshimura *et al.* 1995; Zhang *et al.* 2006). The study of Sharma *et al*. (2004) however, did not show improved

resistance when pyramiding *R*-genes. In this study, marker-assisted pyramiding of the brown planthopper resistance genes *Bph1* and *Bph2* on rice chromosome *12* resulted in a resistance level of the pyramided line equivalent to that of the *Bph1*-single introgression line.

Markers for Marker Assisted Breeding

For breeding companies to breed for introgressed traits such as resistance genes, a selection has to be done. Since the development of DNA marker technology in the 1980's an important alternative for selection with disease tests has become possible. Molecular markers can be used as selection tools and can thus shorten the development of new cultivars (Ribaut and Hoisington 1998). DNA markers can have a couple of advantages over disease testing (Peleman and Rouppe van der Voort 2003). Genotyping with DNA markers can increase reliability, since the environmental effect is taken away from the selection. Genotyping with DNA markers can increase efficiency, since it can be done at the seedling stage. And finally, DNA markers can reduce costs. In high-throughput cases, the costs of PCR screening a large quantity of seedlings will be cheaper than phenotyping them in a disease test. Breeding with the aid of molecular markers is termed Marker Assisted Breeding (MAB)

A range of molecular markers have been developed. They make use of the naturally occurring polymorphisms in the plants' DNA. Molecular markers rely on differences in size of DNA-restriction fragments, in primer binding sites or in the number of repetitive di-, tri- or tetranucleotide units. Recent developments use DNA hybridisation of target DNA with immobilised oligonucleotide sequences on a solid support. Polymorphisms are recognised as differential hybridisation of different fluorescently labelled DNA samples, or absolute hybridisation signal strength. However, to be suitable for MAB, the marker should be low in cost, easy-to-use, robust, reproducible and specific for the desired trait.

The first molecular markers used in plant breeding were Restriction Fragment Length Polymorphisms (RFLPs) (Botstein *et al.* 1980). RFLPs are detected by the use of restriction enzymes that cut genomic DNA molecules at specific nucleotide sequences (restriction sites). When differences in sequences between individuals exist, digestion

with restriction enzymes will yield variable-size DNA fragments. But the RFLP assay is time consuming, labour intensive, and expensive in material use (Powell *et al*, 1996). AFLP marker technology (Vos *et al*. 1995) is reproducible and has the advantage of potentially yielding over 50 markers per run. AFLP markers are used in many genetic studies in potato. But like with RFLP markers, for use in MAB the technical overhead of AFLP markers is too costly. PCR markers like Sequence Characterised Amplified Region (SCAR) markers and Cleaved Amplified Polymorphic Sequence (CAPS) markers are low in cost, robust and convenient in use. For the development of these types of markers prior sequence knowledge is necessary. SCAR markers make use of polymorphisms in the primer sites resulting in an absence or presence of an amplified band, or in differences in fragment length of amplified alleles. CAPS markers have a digestion step after PCR amplification, making use of a restriction site polymorphism. An example of a CAPS marker is shown in Figure 2.

Figure 2: A photographic image of the results of a CAPS marker able to discriminate between resistant (R) and susceptible (S) genotypes.

Objectives and outline of this thesis

This thesis deals with resistance against late blight and nematodes. Besides identifying genes, the effectiveness of pyramiding different genes was assessed.

Chapter 2: A locus involved in late blight resistance, derived from *S. microdontum*, is characterised. Data of a field assay were analysed in a quantitative as well as a

qualitative genetic manner. QTL analysis identified a QTL on chromosome *4* after correction of the resistance data for plant maturity. A qualitative genetic analysis resulted in the positioning of this locus on the short arm of chromosome *4*. A position which coincides with a conserved *Phytophthora R*-gene cluster including *R2, $R_{2\text{-}like}$, $R_{Pi\text{-}blb3}$* and *$R_{Pi\text{-}abpt}$*.

Chapter3: The resistance gene characterised in Chapter 2, *$R_{Pi\text{-}mcd1}$* originating from *S. microdontum* is combined with *$R_{Pi\text{-}ber}$*, originating from *S. berthaultii*, in a segregating diploid *S. tuberosum* population. Individual genotypes from this segregating population were classified into four groups by means of flanking molecular markers. The groups were formed based on: carrying no *R*-gene, with only *$R_{Pi\text{-}mcd1}$*, with only *$R_{Pi\text{-}ber}$*, and a group with the pyramided *$R_{Pi\text{-}mcd1}$* and *$R_{Pi\text{-}ber}$*. The levels of resistance between the groups were compared in a field experiment in 2007. The group with *$R_{Pi\text{-}mcd1}$* showed a significant delay to reach 50% infection of the leaf area of three days. The group with *$R_{Pi\text{-}ber}$* showed a delay of three weeks. The resistance level in the pyramid group suggested an additive higher effect of *$R_{Pi\text{-}mcd1}$* with *$R_{Pi\text{-}ber}$*.

Chapter 4: A resistance to *G. pallida* Rookmaker (Pa3), originating from the wild *Solanum* species *S. tarijense* was identified by QTL analysis. One major QTL, *$GpaXI^{l}_{tar}$* explained 81.3 % of the phenotypic variance in the disease test. *$GpaXI^{l}_{tar}$* mapped to the long arm of chromosome *11*. Another minor QTL explained 5.3 % of the phenotypic variance and mapped to the long arm of chromosome *9*. Clones containing both QTL showed no lower cyst counts than clones with only *$GpaXI^{l}_{tar}$*. After Mendelising the phenotypic data, *$GpaXI^{l}_{tar}$* could be more precisely mapped near markers GP163 and FEN427 thus anchoring *$GpaXI^{l}_{tar}$* to a region with a known *R*-gene cluster containing virus and nematode resistance genes.

Resistance against *Meloidogyne hapla* originating from wild species of tuber-bearing potatoes is almost always non-absolute. In Chapter 5 it was tested if pyramiding of two resistance genes *$R_{Mh\text{-}tar}$* and *$R_{Mh\text{-}chc}A$* will result in a better, or even an absolute level of resistance. *$R_{Mh\text{-}tar}$* and *$R_{Mh\text{-}chc}A$*, introgressed from the wild tuber bearing potato species *S. tarijense* and *S. chacoense* were combined in a segregating diploid *S. tuberosum* population. With the aid of AFLP markers, individual genotypes from this segregating population were classified into four groups, carrying no *R*-gene, with only *$R_{Mh\text{-}tar}$*, with only *$R_{Mh\text{-}chc}A$*, and a group with the pyramided *$R_{Mh\text{-}tar}$* and *$R_{Mh\text{-}chc}A$*.

The pyramiding population was subsequently tested in a disease assay with the *M. hapla* population “Bovensmilde”. The egg masses formed on the root systems were counted and compared between the groups carrying no *R*-gene, only one *R*-gene or both *R*-genes.

In the final chapter, the General Discussion, the results of the preceding chapters are evaluated and the practical implications as well as the fundamental lessons we have learned will be discussed.

Chapter 2

The $R_{Pi\text{-}mcd1}$ locus from *S. microdontum* involved in resistance to *Phytophthora infestans*, causing a delay in infection, maps on potato chromosome *4* in a cluster of NBS-LRR genes

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Abstract

The distinction between field resistance and resistance based on *R*-genes has been proven valid for many plant pathogen interactions. This distinction does not seem to be valid for the interaction between potato and late blight. In this study a locus involved in late blight resistance, derived from *S. microdontum*, provides additional evidence for this lack of distinction. The resistance is associated with a hypersensitive response and results in a delay of infection of about 1-2 weeks. Both a quantitative as well as a qualitative genetic approach were used, based on data from a field assay. QTL analysis identified a QTL on chromosome *4* after correction of the resistance data for plant maturity. A qualitative genetic analysis resulted in the positioning of this locus on the short arm of chromosome *4* in between AFLP marker pCTmACG_310 and CAPS markers TG339 and T0703. This position coincides with a conserved *Phytophthora R*-gene cluster which includes *R2*, $R_{2\text{-}like}$, $R_{Pi\text{-}blb3}$ and $R_{Pi\text{-}abpt}$. This implies that $R_{Pi\text{-}mcd1}$ is the fifth *R*-gene of this NBS-LRR cluster. The implications of our results on *R*-gene based and field resistance are discussed.

Additional keywords: late blight, linkage mapping, potato, *R*-gene, *Solanum microdontum*

Introduction

The disease late blight, caused by the oomycete pathogen *Phytophthora infestans* is a serious worldwide threat to potato cropping. In order to reduce the damaging effects from *Phytophthora*, the use of genetically resistant plants is a potentially viable approach for disease management (Strange and Scott 2005). Eleven resistance genes originating from the wild species *S. demissum* have been discovered in the last century: R1, R2, R3, R4 by Black *et al*. (1953), R5 and R6 by Eide *et al*. (1959), R7, R8 and R9 by Malcolmson and Black (1966) and R10 and R11 by Malcolmson (1969). All these resistance genes offer race-specific hypersensitive resistance but have been proven not to be durable (Malcolmson and Black 1966).

To look for new sources of resistance that could potentially be more durable, researchers have turned to other wild potato species besides *S. demissum*. The resistance gene $R_{Pi\text{-}ber}$ from *S. berthaultii* mapped on chromosome *10* (Ewing *et al.* 2000; Rauscher *et al.* 2006). $R_{Pi\text{-}ber}$ proved to be a new *R*-gene after testing with a set of *Phytophthora* strains capable to identify each of the *R*-genes *R1-R11*. Four *R*-genes have been identified in the wild potato species *S. bulbocastanum*. The *RB* locus has been mapped on chromosome *8* by Naess *et al.* (2000). One allele of this locus has been cloned by Song *et al.* (2003) and another allele, $R_{Pi\text{-}blb1}$ by van der Vossen *et al*. (2003). Another gene that was cloned from *S. bulbocastanum* was $R_{Pi\text{-}blb2}$, located on chromosome *6* (van der Vossen *et al.* 2005). These two genes, *RB*/$R_{Pi\text{-}blb1}$ and $R_{Pi\text{-}blb2}$ have tentatively been described as 'broad spectrum resistance genes'. This new terminology may imply that a larger number of elicitors is being recognized, or may just indicate the current absence of a single compatible race. Finally, two race-specific resistance genes $R_{Pi\text{-}blb3}$ and $R_{Pi\text{-}abpt}$ have been mapped to chromosome *4* (Park *et al.* 2005a; Park *et al.* 2005b).

Besides the dominant *R*-gene based resistance conferring the hypersensitive response (HR), the so called field-resistance is known. This type of resistance is race-non-specific, quantitative and considered as polygenically inherited. It has been assumed that this type of resistance is more durable than *R*-gene based resistance. Race-non-specific foliage resistance is currently differentiated in either maturity corrected resistance (Bormann *et al.* 2004), or maturity associated resistance as was shown by Toxopeus (1958), Van Eck and Jacobsen (1996), Collins *et al* (1999), Oberhagemann *et al* (1999), Costanzo (2005) and Visker *et al*. (2005). The general importance of a locus near marker GP21 on chromosome *5* involved in both maturity and maturity related resistance was reviewed by Simko (2002).

In addition to *S. bulbocastanum* and *S. berthaultii*, the late blight resistance offered by *S. microdontum* has been used in commercial potato breeding during the last decade, although little is known about its inheritance. Resistance derived from *S. microdontum* genotype MCD167 (accession BGRC 24981 / CGN20597) has initially been studied using AUDPC data from a field trial (Sandbrink *et al.* 2000). Their analysis of the quantitative inheritance of this resistance embarked on two assumptions: (1) that the *S. microdontum* resistance was polygenic and (2) conferred race-non-specific resistance. Sandbrink *et al*. (2000) identified a major QTL on

chromosome *4* explaining 40% of the phenotypic variation, and two QTLs on chromosome *5*. An additional major QTL was identified in the same study in genotype MCD 178 (accession BGRC 24981 / CGN20597). Bisognin *et al*. (2005) have also described a field resistance derived from *S. microdontum* (BGRC 27353 / CGN20640), but the linkage group containing the only QTL could not be assigned to a specific potato chromosome. Lastly, it is known that the foliage resistance observed in *S. microdontum* BGRC 18302 (CGN21342) is also effective in the tuber (Park *et al*. 2005d)

In this paper we describe the genetic and phenotypic analysis of late blight resistance derived from *S. microdontum*. Our plant material represents a different accession than the accessions described by Sandbrink and Bisognin. The accession used in this study is also used in commercial breeding programs in the Netherlands and consistently displays a 1-2 week delay in infection in the field (unpublished data). Furthermore we have mapped our locus on chromosome *4* and narrowed down its position to a conserved *Phytophthora R*-gene cluster which includes *R2*, $R_{2\text{-}like}$, $R_{Pi\text{-}blb3}$ and $R_{Pi\text{-}abpt}$.

Materials & Methods

Plant Material

A diploid interspecific mapping population RH94-076 (n=224) was created with a cross between RH90-038-21 and RH88-025-50. The pedigree of RH94-076 is presented in Figure 1. Because of the susceptible back-cross parents and the agronomical selection in the BC1 offspring, this BC2 mapping population should be regarded as essentially a susceptible *S. tuberosum* genetic background with less than 12.5 % *S. microdontum* introgression. The *S. microdontum* accession BGRC 18302 (CGN21342) was collected by Hjerting, during a Danish expedition (HPR 0293) in 1965/66 to Argentina.

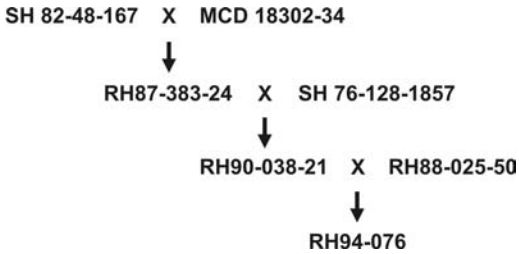

Figure 1: Pedigree of the *S. microdontum* derived mapping population "RH94-076", derived from accession BGRC 18302, backcrossed with susceptible diploid *S. tuberosum* clones.

***In vitro* assay**

371 newly sown genotypes of RH94-076 were tested in an *in vitro* assay as described by Huang *et al*. (2005) with *Phytophthora infestans* isolate 90128 (race 1, 3, 4, 6, 7, 8, 10, 11) provided by Dr. F. Govers, Laboratory of Phytopathology, Wageningen University, The Netherlands. Symptoms were recorded at 3, 4, 5, 6 and 7 days after infection. Sporulation or large scale necrosis was interpreted as susceptible. A hypersensitive response (HR) or lack of symptoms was interpreted as resistant. The susceptible cultivar Bintje and the $R_{Pi\text{-}abpt}$ containing resistant genotype 707TG11-1 (Park *et al.* 2005a) were used as control plants.

Detached leaf assay

Leaves collected from greenhouse plants of 96 genotypes of RH94-076 were tested in four replications with a detached leaf assay (Vleeshouwers *et al.* 1999). Parents RH90-038-21 and RH88-025-50 were included as control plants. Complex isolate 90128 (race 1, 3, 4, 6, 7, 8, 10, 11) was used to determine leaf resistance. Inoculation was performed as described in Vleeshouwers *et al*. (1999) except that the concentration of the inoculum was adjusted to 5×10^5 zoospores/ml.

Field-test 2001

The field test was performed in 2001 on 224 genotypes of RH94-076. The location of the field was the Meenthoeve, in the area near Wageningen, NL, on sandy soil. Tubers were planted at the 15th of May 2001. Experimental design was a randomized block design. Four tubers per genotype were planted. The four plants per genotype were treated as one experimental unit. Inoculation was performed with a spore suspension of a complex *P. infestans* isolate IPO-82001 (race 1, 2, 3, 4, 5, 6, 7, 10, 11; provided by W. Flier, Plant Research International) as described by

Vleeshouwers *et al*. (1999) on the 11th July. Because of a lack of infection the field was re-inoculated with the same isolate on 17th July. Infection levels were scored 12 times, every other day, over a period of 3.5 weeks as the average leaf area covered by lesions (as a percentage of the total leaf area). Observation time points are indicated as days post infection (dpi) taking 17th July as day zero. Values of rAUDPC were calculated as described in Fry (1978).

Maturity type was scored on a fungicide protected field experiment in the 3rd week of August on an ordinal scale ranging from 2 (green and vigorous; late to very late) to 6 (yellowing and plant is sagging) to 10 (dead; very early plant types).

Classification of foliage infection values into resistant / susceptible phenotypes

Field test observations from 2001 were converted into a tentative phenotypic classification of descendants with or without resistance factors, taking the observations at 7-13 dpi. This classification used the population mean as threshold. Offspring with infection lower or higher than the population mean were classified as resistant or susceptible, respectively.

DNA extraction

Genomic DNA of the individuals of mapping population RH94-076 and parents RH90-038-21 and RH88-025-50 was isolated from young leaf material as described by Van der Beek *et al*. (1992). Tissue was ground with steel balls using a Retsch machine (Retsch Inc., Haan, Germany) in the STE-buffer as described, with volumes adapted to 2 ml deep 96-wells Costar plates (Corning Inc., Corning, NY, U.S.A.).

AFLP analysis

AFLP™ analysis was performed according to Vos *et al*. (1995). Twelve primer combinations were used: E+AAC/M+CAC, E+AAC/M+CAG, E+AAC/M+CCA, E+AAC/M+CTG, E+AAT/M+CGA, E+ACA/M+CAA, E+ACA/M+CAC, E+ACA/M+CCT, E+ACA/M+CGT, E+AGA/M+CTC, E+ATG/M+CAG, E+ATG/M+CTC. AFLP fragments were visualized on radioactive gels and scored visually for absence or presence. AFLP marker names are composed of the restriction enzyme combination followed by the three selective nucleotides of the primers followed by the migration on gel relative to the 10-base ladder (Sequamark,

Research Genetics). Marker names with sizes in tenths of nucleotides refer to markers mapped before in reference populations (http://www.dpw.wau.nl/pv/aflp/catalog.htm and http://potatodbase.dpw.wau.nl/UHDdata.html). These reference markers can be used to assign linkage groups to potato chromosomes. The other markers are unique for this material.

Map construction

With JoinMap (van Ooijen and Voorrips 2001) 210 AFLP markers were assigned to linkage groups using a LOD threshold of 5.0 for grouping. In a second version of the map 32 SSR markers were included for confirmation of chromosome identity. The entire map is composed of 12 maternal and 12 paternal linkage groups, with 26 markers remaining unassigned. Details on the map and map construction will be published elsewhere (Werij *et al*., manuscript in preparation). The tentative classification of late blight resistance phenotypes, described above, was used as marker data of the $R_{Pi\text{-}mcd1}$ locus and included in map construction.

Bulked Segregant Analysis

Bulked Segregant Analysis (Michelmore *et al.* 1991) was applied to identify additional markers in the proximity of the locus involved in resistance, with the aim to further saturate the genetic map. Four bulks were composed as follows: Br and Bs each containing eight non-recombinant genotypes with a resistant or susceptible phenotype respectively, and two additional bulks, Brc (n=5) and Bsc (n=8) were composed to narrow down the target window on chromosome *4*, using descendants with cross-over events between markers flanking the locus involved in resistance. The cross-over events were inferred between AFLP markers eAGAmCTC_155 and eATGmCAG_187.2 with a distal position, as known from our marker catalogue. Bulks were analysed with 256 PstI+NN/MseI+ANN and 256 PstI+NN/MseI+CNN primer combinations. Fluorescently labelled AFLP fragments were visualized on a denaturing polyacrylamide gel using a NEN® Global Edition IR2 DNA Analyzer (LI-COR® Biosciences, Lincoln, NE). AFLP primer combinations using *Pst*I/*Mse*I template were used, because the chromosomal distribution of *Pst*I/*Mse*I compares favourably to *Eco*RI/*Mse*I derived AFLP markers. The majority of *Eco*RI/*Mse*I derived AFLPs cluster in centromeric regions as shown by Van Os *et al*. (2006).

Excision of AFLP fragments

*Pst*I primer PstI+GT was radioactively labelled with ^{33}P. AFLP was performed according to (Vos *et al.* 1995). AFLP bands of marker pGTmACG _310 of 8 samples: 2 x RH90-038-21, 2 x R88-250-50 + 4 susceptible individuals were excised from gel and dissolved in 50 µl water. Supernatant was re-amplified with P+0 and M+A primers and purified with a G50 column before sequencing. pGTmACG_310 sequence was Blastn on URL www.ncbi.nl.nih.gov/BLAST .

CAPS markers

Further anchoring of the resistance locus was achieved with chromosome *4* specific markers TG339, CT229 and T0703. PCR primers were designed using sequence information from SOL Genomics Network (www.sgn.cornell.edu) with Primer3 software (Rozen and Skaletsky 2000). PCR-products were digested with 27 restriction enzymes to identify polymorphic sites. The potato version of TG339 was developed using GenBank accession number X87370 (*Solanum tuberosum* mRNA for 14-3-3 protein) obtained with Blastn using the original tomato TG339 RFLP sequence. The TG339 primers are F:GCTGAACGCTATGAGGAGATG / R:TGAGGTTATCACGCAGAAGTTG. The CAPS polymorphism was obtained by digestion with *Mnl*I. Marker CT229 was amplified with primers F:TTGTGAGTGGTGAACTACGGGC / R:CGGCAATGGTTATGGGAACG (Park *et al*. (2005b), and displayed a CAPS polymorphism using restriction enzyme *Hpy*CH4IV. The sequence of tomato COS marker T0703 was used for conversion into a CAPS marker. Primers for the T0703 marker are (F:CCAGTAAGAACAAGCCGATT / R:ATCACCAATTACGCGATCTA). A polymorphism was obtained upon restriction with *Bme*1390I.

Chromosome *4* map construction

The marker order of maternal linkage group *4* including the added AFLP markers from BSA and CAPS markers TG339, CT229 and T0703 was re-assessed with RECORD (van Os *et al.* 2005a) and visually inspected by graphical genotyping the raw data in MS-Excel using the conditional formatting of cells conditional to marker genotype and linkage phase. Marker pairs without obvious recombination events were placed at the same map position. This avoids suggestive marker orders without clear data support. Final map length of the linkage group including $R_{Pi\text{-}mcd1}$ reflects the sum of adjacent recombination frequencies (Stam 1993)

QTL analysis

QTL analysis was performed with MapQTL 5 (van Ooijen 2004) using a linkage map of 1179.2 cM (maternal plus paternal map length). An initial search for QTLs was performed with Kruskal-Wallis as no assumptions of normality were made. For interval mapping purposes the average leaf area covered by lesions (%) per observation and per genotype was converted to degrees by the variance-stabilising angular transformation (angle whose sine is the square root of the percentage of foliage infection).

Results

In vitro and detached leaf assay

Because of its efficiency and previous successes, the *in vitro* late blight resistance assay (Huang *et al.* 2005) was used to classify 371 seedlings of mapping population RH94-076. The susceptible control Bintje sporulated after 3 days. The resistant control 707TG11-1 showed a hypersensitive response (HR) and no sporulation. Most of the genotypes of RH94-076 were sporulating within 7 days. 128 genotypes sporulated without a HR. 186 genotypes showed a weak incomplete HR before sporulation. Twelve genotypes showed no symptoms at the end of the observation period and 45 genotypes gave a HR and no sporulation, so those 57 could be considered as resistant. These results indicated that some form of resistance is present. Based on these observations unambiguous classification of seedlings as resistant or susceptible was impossible. The most important problem is that no apparent Mendelian segregation ratio could be identified, preventing a Bulked Segregant Analysis approach. For these reasons we proceeded with a detached leaf assay.

In the detached leaf assay, the *S. microdontum* derived BC1, parent RH90-038-21 was resistant showing HR and the *S. tuberosum* parent RH88-025-50 was susceptible. However, clones of the mapping population showed inconsistent phenotypes where sometimes only one or two leaves out of the four leaves per genotype were infected and showed sporulation, whereas the other ones were without symptoms. When the entire detached leaf assay was repeated, again with four leaves per genotype, the newly obtained results poorly correlated with the first

assay. The phenotypic differences observed among the progeny displayed a continuous distribution and could not be differentiated in a resistant or susceptible class. The resistance derived from *S. microdontum* appeared to be quantitative in nature, which could be much better evaluated using a field experiment. In summary, the value of the *in vitro* assay and the detached leaf assay is limited, and only allows to conclude that these tests require plant material with strong effect *R*-gene(s), as shown before (Huang *et al*, 2005; Vleeshouwers *et al*. 1999).

Quantitative genetic analysis of disease resistance and plant maturity

The mapping population, consisting of 224 clones, was evaluated for the level of resistance in a field test in 2001. Observations ranged from 7 dpi until 29 dpi. When rAUDPC was used as trait value in Kruskal-Wallis based QTL detection, three QTL peaks were identified near markers eATGmCAG_235 (at chromosome *4*), eACAmCAC_19 and Sti032 (both at chromosome *5*) with K*-values of 78, 28 and 24 respectively. When percentage of infected foliage at a given day was used as trait value, higher K*-values were obtained, with QTL peaks at the same three markers.

The most prominent QTL was identified by any of the markers along maternal chromosome *4*. AFLP marker eATCmCAG_235 identified this QTL irrespective of observation time during the whole time range of the experiment. The percentage of infected foliage at 9-11 dpi was the most informative trait value, showing the highest K*-values (K* = 90-91) at AFLP-marker eATGmCAG_235. At these days (9-11 dpi) all associations between the trait value and the markers of chromosome *4* reached significance levels of $p < 0.0001$, except for the most distal marker eATGmCAG_187 ($p < 0.005$). Figure 2 illustrates the position of the Kruskal-Wallis identified QTL peak at 11 dpi. With interval mapping using the angular transformed data, the explained variance was 50.3% at a LOD of 34, indicating a QTL with a large effect, located on chromosome *4*. From 13 dpi the test statistic lowered gradually from K*86 at 13 dpi to K*31 at 29 dpi (end of observation). At 29 dpi the QTL-effect resulted in an explained variance of 5.5 % at a LOD of 2.88. Observations later than 11 dpi did not offer the same power to detect this QTL, because at later observations an increasingly larger part of the population was completely infected.

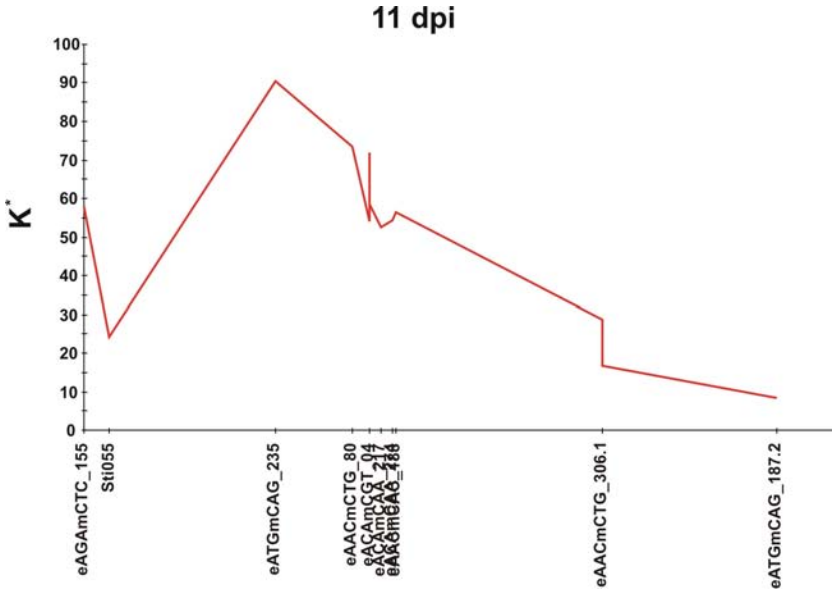

Figure 2: Kruskal-Wallis curve showing the QTL peak at the position of marker eATGmCAG_235 on chromosome *4* of *S. microdontum parent* (BC1) RH90-038-21, marking a locus with a major effect on percentage of leaf area infected by *P. infestans*.

Two other QTLs involved in percentage foliage infection were found, namely on chromosome *5* of the maternal and the paternal map. The QTL on maternal chromosome *5* of RH90-038-21 had significant ($p < 0.0001$) association with 2 AFLP markers and Sti032 marker with a K* value of 17 increasing gradually to the highest value K*32 at 25 dpi. With interval mapping, the QTL increases gradually to the highest value of LOD 8.85 with an explained variance of 16.2% at 25 dpi. The QTL on paternal chromosome *5* of RH88-025-50 had significant ($p < 0.0001$) association with 3 AFLP markers and SSR markers STM5148 and Sti032. From 7 dpi the K* value of 19 increased gradually to the highest K* 39 value at the final observation date. With interval mapping this QTL reaches the highest LOD 6.36 at 27 dpi (11.6%)

When observations for maturity type were used as trait value in MapQTL two K* peaks were observed near markers eACAmCAC_19 (K* = 31) and Sti032 (K* = 41) on paternal and maternal linkage group *5*, respectively. Because on female and male chromosome *5*, the QTLs involved in percentage infected foliage and maturity type

coincided, we proceeded with QTL analysis using maturity corrected resistance (MCR) (Bormann *et al.* 2004; Visker *et al.* 2004). This trait value represents late blight resistance compensated for the effect of maturity, and was obtained by subtracting maturity values from percentage foliage infection, leaving the residuals. MapQTL analysis using the residuals (MCR) resulted in the detection of the same QTL on chromosome *4* with equal significance, but the QTLs on chromosome *5* were no longer detected ($p > 0.05$).

In conclusion, among the initial three QTLs, we deduced that the large effect QTL near marker eATGmCAG_235 on chromosome *4* of the resistant parent is a major effect QTL involved in *Phytophthora* resistance. The smaller effect QTLs, both residing on chromosome *5* of either the susceptible and resistant parent, are QTLs involved in plant maturity, and affect late blight infection indirectly.

Qualitative genetic analysis of resistance

Besides quantitative trait analysis an attempt was made to categorise offspring clones in a resistant and susceptible class. The disease progress curves of each offspring genotype are shown in Figure 3. This figure suggests that the most clear-cut classification can be made at 11 dpi.

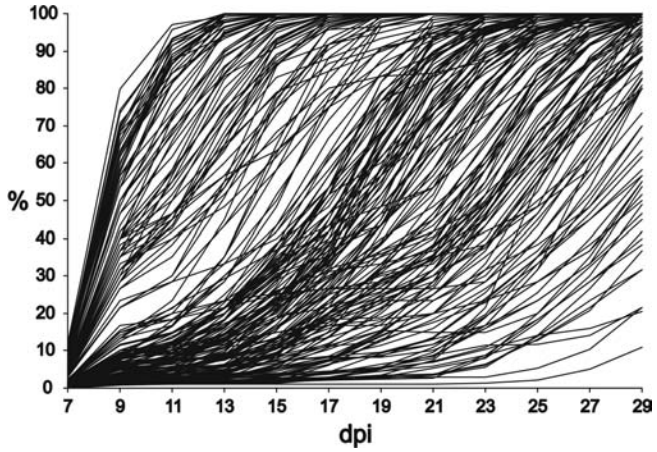

Figure 3: Late blight disease progress curve in percentage of leaf area infected across 23 days post infection (dpi) of 244 offspring clones of *S. microdontum* BC2 population RH94-076.

The distribution at 11 dpi is presented in Figure 4. At 11 dpi the susceptible group had an average foliage infection of 64% while the resistant group was on average 7% attacked. On the last day of observation (29 dpi), the susceptible group was 100% infected and the resistant group had an average foliage infection of 88%, using the tentative classification of the offspring at 11 dpi. The 11 dpi classification resulted in a segregation of 150 resistant and 74 susceptible genotypes, which is a significant deviation from a 1:1 ratio (χ^2 = 25.8; p<0.0001). Linkage analysis with this locus representing late blight resistance along with the molecular markers, resulted in the construction of a linkage group with a locus named $R_{Pi\text{-}mcd1}$ (Figure 5). The mapping results of $R_{Pi\text{-}mcd1}$ allowed to validate the qualitative genetic analysis of late blight resistance, because the expected resistance genotype, based on flanking markers, could be compared with the observed phenotype based on tentative classification. Only 3 false resistant phenotypes (clones 59, 187, 232) and 7 false susceptible phenotypes (clones 20, 21, 84, 135, 140, 264, 276) among 224 offspring classifications were identified (Figure 4). All of these false resistant or susceptible classifications belonged to genotypes without recombination event in linkage group *4*, thus could not interfere with the position of $R_{Pi\text{-}mcd1}$ on the short arm of chromosome *4*. With 13 dpi, three additional resistant plants were incorrectly scored as susceptible, and one susceptible plant was incorrectly scored as resistant.

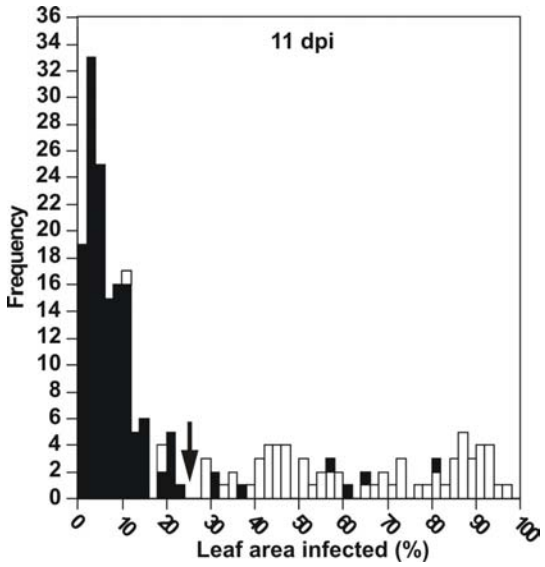

Figure 4: Histogram showing the frequency distribution of percentage leaf area infected by late blight at 11 days post infection, the observation date which gives the most clear-cut division between resistance and susceptibility. The arrow indicates the cut-off value (the average population infection at 11 dpi (25.7 %)). Genotypes to the left of the arrow were classified as resistant, genotypes to the right were classified as susceptible. Black = $R_{Pi\text{-}mcd1}$ present according to flanking markers, white = $R_{Pi\text{-}mcd1}$ not present according to flanking markers.

Based on the absence or presence of $R_{Pi\text{-}mcd1}$ in the offspring, the two average disease progress curves could be calculated. These average curves clearly differ in slope. At 50% loss of leaf tissue the infection rates were 8.4 % (without $R_{Pi\text{-}mcd1}$) and 5.1 % (with $R_{Pi\text{-}mcd1}$) increase of foliage infection per day. The average difference in time to reach the level of 50% leaf infection was 11.3 days between genotypes without $R_{Pi\text{-}mcd1}$ compared to genotypes with $R_{Pi\text{-}mcd1}$.

Anchoring of $R_{Pi\text{-}mcd1}$ and saturation of the chromosome *4* linkage map (Figure 5)

The identity of the linkage group comprising the QTL and/or $R_{Pi\text{-}mcd1}$ locus was first indicated by several AFLP markers which were known chromosome *4* specific markers (http://potatodbase.dpw.wau.nl/UHDdata.html). This was confirmed when RFLP marker TG339, converted into a CAPS marker, mapped six recombination events (2.8 cM) below $R_{Pi\text{-}mcd1}$ (towards the centromere) as is shown in Figure 5.

Bulked Segregant Analysis with 256 primer combinations of PstI+NN/MseI+ANN resulted in five bulk specific bands of which two were false positive bands, two markers pCTmAGC_378.0 and pCTmACG_90 mapped in coupling phase and marker pCTmACG_310, in repulsion phase, showed complete cosegregation with $R_{Pi\text{-}mcd1}$. The second BSA with 256 primer combinations PstI+NN/MseI+CNN did not result in additional markers.

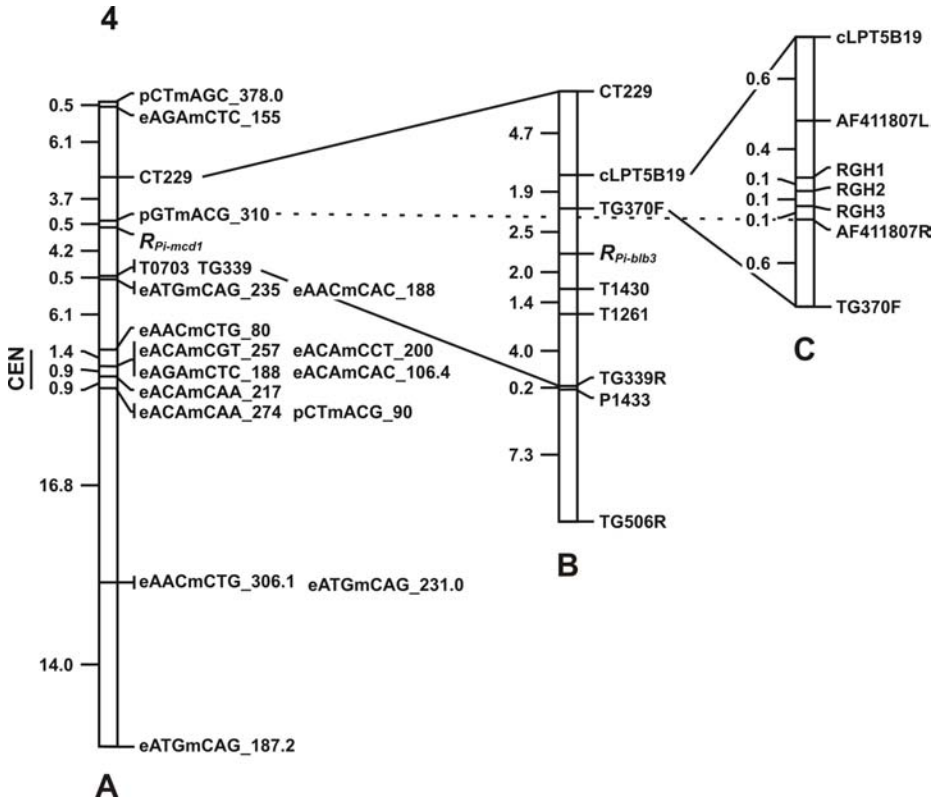

Figure 5: $R_{Pi\text{-}mcd1}$ maps on chromosome *4* to a known cluster of resistance genes and NBS-LRR resistance gene candidates. A. Genetic map of chromosome *4* of the maternal resistant parent RH90-038-21 showing the location of $R_{Pi\text{-}mcd1}$. CEN = centromere location. B: Genetic map of a part of chromosome *4* with the location of $R_{Pi\text{-}blb3}$ (Park *et al.* 2005b). C: High resolution map of the chromosome *4* interval distal to $R_{Pi\text{-}blb3}$ with the genetic position of CAPS marker AF411807L and AF411807R based on the BAC end sequences, and three *R*-gene homologs (RGH1-3) of tomato BAC AF4011807. AFLP marker pGTmACG_310 from this study was identified on BAC AF411807 near the position of AF411807R.

Tomato COS marker T0703, converted into a CAPS marker, mapped at the same position as TG339. Tomato RFLP marker CT229, converted into a CAPS CT229, mapped nine recombination events (4.2 cM) distal of $R_{Pi\text{-}mcd1}$. By the addition of this marker, marker pGTmACG_310 did no longer co-localize with $R_{Pi\text{-}mcd1}$; a genotype which previously was thought to have a singleton event, proved to be a true recombinant, with the recombination between marker pGTmACG_310 and $R_{Pi\text{-}mcd1}$.

AFLP marker pGTmACG_310 was excised from gel, sequenced and a Blastn search resulted in strong homology to tomato BAC AF411807 (van der Hoeven *et al.* 2002), which was assigned to chromosome *4* of tomato between markers CT229 and TG370 (Tanksley *et al.* 1992). The sequence of marker pGTmACG_310 was aligned with vector NTI to position 95735-95837 of BAC AF411807 which is close to the R- end of this 95845 bp long BAC sequence**.**

The segregation pattern based on the classification using 11 dpi, positioned $R_{Pi\text{-}mcd1}$ proximal of marker pGTmACG_310 on the graphical genotype map, with one recombination event between marker pGTmACG_310 and $R_{Pi\text{-}mcd1}$ at a distance of 0.5 cM. $R_{Pi\text{-}mcd1}$ was positioned at a distance of nine recombination events (4.2 cM) distal to the flanking markers T0703 and TG339. $R_{Pi\text{-}mcd1}$ appeared to be located on the short arm of chromosome *4*. Centromere location was inferred from the clustering of *Eco*RI/*Mse*I markers around the centromere (Park *et al.* 2007; van Os *et al.* 2006), and from tomato and AFLP anchor markers.

Validation of CAPS markers

CAPS markers TG339, CT229 and T0703 were tested for *S. microdontum* specific polymorphism in a range of germplasm (n=96) comprising DNA of *S. tuberosum, S. berthaultii, S. microdontum, S. bulbocastanum, S. tarijense, S. fendleri, S. hougassi,* and *S. vernei*. None of the marker alleles were exclusively diagnostic for $R_{Pi\text{-}mcd1}$. Neither were these markers specific for wild germplasm and absent in cultivated potato. Therefore these markers in combinations with these restriction enzymes, which work well for this mapping population, can not be used for marker assisted breeding in a wider range of germplasm.

Discussion

In this paper a range of *Phytophthora* assays has been used that are usually not applied in combination on the same material. Although the *in vitro* and detached leaf assays did not contribute to the localisation of $R_{Pi\text{-}mcd1}$, the joint results allowed us to characterise the phenotype of $R_{Pi\text{-}mcd1}$ as a locus involved in late blight resistance, and to evaluate our results in relation to current models on late blight resistance.

The *in vitro* assay resulted in inconclusive data, because most of the genotypes of the *S. microdontum* mapping population were sporulating within 7 days after showing a weak HR. With the detached leaf assay, clones of the mapping population showed inconsistent phenotypes. These results indicated that some form of resistance should be present in this material, but a qualitative genetic interpretation of the in the *in vitro* and detached leaf assays was not obvious. For a quantitative interpretation, data were subsequently collected from a field assay. When using rAUDPC and percentage foliage infection as trait values, three QTLs were detected. One major QTL associated with resistance was located on chromosome *4*. The two other QTLs were detected on the maternal and paternal linkage groups corresponding to potato chromosome *5*. However, when the trait values were corrected for maturity, QTLs on chromosome *5* could no longer be detected. This indicates that the QTLs of chromosome *5* were involved in plant maturity.

The rAUDPC is currently the most accepted trait value to assess resistance levels. In this study however, the best trait value for QTL analysis proved not to be the rAUDPC but percentage foliage infection at 9-11 dpi. The latter method showed the highest association in K*-values in QTL analysis. The explanation for the lower power of rAUDPC as trait value in QTL identification can be the effect of inclusion of too late and thus indiscriminative observations within the highly informative observation at the beginning.

The best approach to localise hereditary factors however, is not QTL analysis at all, but Mendelian classification. Classification of the foliage infection values resulted in accurate localisation of $R_{Pi\text{-}mcd1}$ on chromosome *4*. At this genetic position only 10 false positives/ false negatives were inferred using markers flanking $R_{Pi\text{-}mcd1}$ among 224 offspring. Timing is important, because the observations at 7-11 dpi - the early start of infection - resulted in a reliable classification. When later time points were used, additional genotypes resulted in misclassification (based on the flanking markers) as resistant or susceptible. Classification results in a single genetic position, whereas the QTL peak (Sandbrink *et al.* 2000) occupies a much larger genetic interval. It can not be excluded that the resistance QTL on chromosome *4* by Sandbrink *et al*. (2000) is identical to the locus mapped in this study, although a different accession, BGRC 24981, was used.

Marker saturation with BSA resulted in the identification of marker pGTmACG_310 at a distance of 0.4 cM of $R_{Pi\text{-}mcd1}$. The DNA sequence of this marker showed strong homology to a part of tomato BAC AF411807, which had been identified in a previous study (Park *et al.* 2005a). Park *et al*. identified the same BAC-clone with AFLP marker pATmAGA_307 which was tightly linked (< 0.1 cM) to another late blight resistance gene $R_{Pi\text{-}abpt}$ on chromosome *4*. On this BAC-clone three NBS-LRR genes were identified with high homology to AFLP marker pATmAGA_307 which also has an NBS-LRR sequence. Currently, three more resistance genes *R2*, *R2-like* and $R_{Pi\text{-}blb3}$ have been mapped along with $R_{Pi\text{-}abpt}$ in this chromosome *4* *R*-gene cluster (Li *et al.* 1998; Park *et al.* 2005a; Park *et al.* 2005b; Park *et al.* 2005c). Our data indicate that $R_{Pi\text{-}mcd1}$ is identified as the fifth member of this *R*-gene cluster.

Strong indication that $R_{Pi\text{-}mcd1}$ belongs to the family of NBS-LRR genes

In our view, the mapping of $R_{Pi\text{-}mcd1}$ in a well known *R*-gene cluster of NBS-LRR genes is the most fascinating result of this study. Theoretically, we cannot exclude that the gene underlying the $R_{Pi\text{-}mcd1}$ locus does not belong to the family of NBS-LRR genes. But other projects in our lab (personal communication Edwin van der Vossen) suggest a limited genetic and physical size of the region on potato chromosome *4*, comprising the series of NBS-LRR genes underlying the $R_{Pi\text{-}blb3}$, *R2*, *R2-like* and $R_{Pi\text{-}abpt}$ loci. Thus it appears that it is more plausible to accept the hypothesis that $R_{Pi\text{-}mcd1}$ is a member of an allelic series of *R*-genes, than to expect an entirely different kind of gene, although supporting information is required to arrive at a final conclusion.

Embarking from the hypothesis that $R_{Pi\text{-}mcd1}$ is an NBS-LRR gene we should now discuss the phenotypic effects observed for $R_{Pi\text{-}mcd1}$ in comparison with the effects associated with NBS-LRR based *R*-genes involved in late blight resistance. To this end we first need to give an overview of the different models of the potato – late blight interaction, as they have appeared in the scientific literature (see Table 1).

Table 1: Overview of various models used in literature to describe the potato – late blight interaction in qualitative and quantitative genetic terms.

Model	Genes	Literature reference
R-gene based resistance (where often monogenic *R*-gene based resistance is implied)		
1. Non-durable race specific resistance	*R1*-*R11*	(Black *et al.* 1953; Eide *et al.* 1959; Malcolmson 1969; Malcolmson and Black 1966)
2. Broad spectrum resistance (race non-specific resistance)	*RB*, $R_{Pi\text{-}blb1}$	(Song *et al.* 2003; van der Vossen *et al.* 2003)
3. Residual resistance	*R1*, *R10*, *R11*	(Stewart *et al.* 2003)
QTL based resistance (where often a polygenic and non-*R*-gene based resistance is implied)		
4. Field resistance in *R*-gene free cultivars		(Colon *et al.* 1995; Landeo *et al.* 1995; Turkensteen 1993)
5. QTLs involved in resistance (with or without correction for plant maturity)	Various QTLs	(Collins *et al.* 1999; Leonards-Schippers *et al.* 1994; Oberhagemann *et al.* 1999); (Bormann *et al.* 2004; Visker *et al.* 2003)

Different models of potato – late blight interaction

The most intriguing distinction between late blight resistance models is based on *R*-gene based and non-*R*-gene based resistance. This distinction is of primary importance, because in general *R*-gene based resistance is assumed to be race-specific and not durable, whereas non-*R*-gene based resistance should be durable, race-non-specific, and thus valuable (van der Plank 1968). The *R*-gene based resistance is based on monogenic factors. The first resistance model in Table 1 "non-durable race specific resistance" refers to the *S. demissum* derived *R*-genes which were soon defeated once cultivars were grown at larger scale. Although lumped in one model, there are differences between the effects of these *R*-genes. Some *R*-genes are known as stronger effect (*R1*, *R3*) or weaker effect *R*-genes (*R10*). Furthermore, although defeated, *S. demissum R*-genes such as *R2* still offer protection in certain growing areas (Pilet *et al.* 2005) which might be explained by a proportion of the

P. infestans population participating in the epidemic carrying the avirulence factor to *R2*. Although increased deployment of cultivars with *R2* is unlikely to make a lasting contribution to late-blight control.

The second resistance model "broad spectrum resistance" refers to *S. bulbocastanum*- derived resistance (Song *et al.* 2003; van der Vossen *et al.* 2003; van der Vossen *et al.* 2005). Because this resistance is based on the usual NBS-LRR type *R*-gene, we assume that broad spectrum only indicates the current absence of a compatible race. There is no reason to assume that the durability of these newly deployed *R*-genes would differ from the durability experienced in the 20th century with *S. demissum* derived resistance. Therefore, we anticipate that our 1st and 2nd model are not essentially different.

The third model "residual resistance" refers to the contribution of defeated *R*-genes to the quantitative level of field resistance (Durel *et al.* 2003; Pedersen and Leath 1988; Stewart *et al.* 2003). A classical example of a residual effect of defeated *R*-genes has been presented for various *Xanthomonas* resistance genes in rice (Li *et al.* 1999). This term should not be confused with the horizontal resistance observed after vertical resistance has been defeated (van der Plank 1968). Here, the residual effect is ascribed to the defeated *R*-gene itself (Durel *et al.* 2003). Therefore, residual resistance should not be regarded as a different model but an asset of the normal *R*-genes belonging to the first model.

The fourth model "field resistance in *R*-gene free cultivars" was historically pursued in response to the lack of durable *R*-genes. Using old potato cultivars, Colon *et al*. (1995) observed that current resistance ratings correlated well with ratings given between 1929 and 1954. This suggests that old potato cultivars, free from *R*-genes, offer a stable and heritable source for resistance. Although Colon *et al*. (1995) have noticed the correlation between plant maturity and late blight resistance, they suggested the presence of durable resistance, acting in addition to the maturity effect (Toxopeus 1958). The stability of resistance to *Phytophthora infestans* in old cultivars without *R*-genes (Colon *et al.* 1995; van der Plank 1971) resulted in a new breeding strategy as promoted by Turkensteen (1993) and Landeo *et al*. (1995). However, stable field resistance as perceived by Van der Plank and Colon *et al*. in old potato cultivars, is to our opinion nothing but the effect of plant maturity, and

perhaps the residual effect of indigenous *S. tuberosum R*-genes. This view was also strongly advocated by Allefs *et al*. (2005).

The fifth model "QTLs involved in resistance" is represented by a number of studies that were among the first to fully exploit the molecular genetic tools and QTL analysis. These studies embarked in general on the assumptions, that resistance is phenotypically quantitative, race-non-specific and possibly polygenically inherited. The choice of the plant material in these studies was largely influenced by the desire not to re-identify *R*-genes, but loci involved in partial, field or horizontal resistance. The major QTLs identified by the studies of Leonards-Schippers *et al*. (1994), Collins *et al*. (1999) and Oberhagemann *et al*. (1999) were always on potato chromosome *5* and coincided with a QTL for maturity type. In our opinion the major QTL involved in resistance are essentially the same as the loci involved in field resistance of the fourth model (van Eck and Jacobsen 1996). Current methods of data analysis include maturity as co-variable during QTL analysis. Alternatively, resistance data are corrected for plant maturity (maturity corrected resistance, MCR), hence leaving the residual variance as trait value (Bormann *et al.* 2004; Visker *et al.* 2003). In view of our remarks on the fallacy of field resistance in *R*-gene free material, we assume that MCR is caused by genes that belong to the NBS-LRR family.

Sifting through these views on the potato – late blight interaction, we construe that in essence, resistance is determined by two factors only: plant maturity and *R*-genes that belong to the NBS-LRR family. The latter factor can be subdivided in stronger and weaker effect *R*-genes, broad spectrum and race specific *R*-genes, broken *R*-genes with larger or smaller residual effects, etc. Nevertheless, all these classes are to be ascribed to NBS-LRR genes and the underlying elicitor – receptor model (Allefs *et al.* 2005; Kamoun *et al.* 1999).

Evaluation of $R_{Pi\text{-}mcd1}$ in relation to current models of potato – late blight interaction

Having described: (1) the phenotypic effects of $R_{Pi\text{-}mcd1}$ in different assays, (2) the quantitative and qualitative genetic analysis of the resistance, and (3) its genetic position in an *R*-gene cluster on potato chromosome *4*, we now wish to evaluate $R_{Pi\text{-}mcd1}$ in relation to the models of the potato – late blight interaction described above.

Our quantitative genetic analysis of $R_{Pi\text{-}mcd1}$ resulted in explaining 50% of the phenotypic variance. This suggests partial resistance, as described in our 4th and 5th model, but our phenotypic observations disagree with the commonly held views on partial resistance. By definition, partial resistance results in a reduced epidemic built-up of the pathogen, notwithstanding a susceptible, non-hypersensitive infection type (Parlevliet and van Ommeren 1975). Partial resistance, due to genes with small effects is thought not to be based on receptor-elicitor recognition (McDonald and Linde 2002). The effect of $R_{Pi\text{-}mcd1}$ is associated with an HR, followed by sporulation. The HR is not in accordance with the definition of partial resistance, but fits closer with observations described by Vleeshouwers *et al*. (2000) where the quantitative nature of *P. infestans* resistance is explained by differences in timing of HR induction and differences in percentage of cells displaying HR after infection, possibly due to inadequate or delayed recognition of elicitors by weak-effect *R*-genes. Their histological studies also indicated that at the cellular level, the difference between compatible and incompatible interactions was quantitative rather than qualitative in nature. This may result in phenotypes which have been described by terms such as: restricted lesion, a restricted but sporulating lesion, a spreading and sporulating lesion. In particular the second table of Vleeshouwers *et al*. (2000) demonstrates that HR is observed during all forms of potato – late blight interaction (susceptible, R-gene based and non-host resistance). Hence HR and successful infection are not a paradox.

Our *S. microdontum* derived BC2 mapping population was susceptible in the *in vitro* assay, susceptible in the detached leaf assay and showed a delayed infection in a field assay. The *in vitro* assay is known to be very efficient, but also very rigorous and will only allow the identification of *R*-genes with strong effects, i.e. which always show full resistance to avirulent *P. infestans* isolates (Huang *et al.* 2005). Likewise the detached leaf assay is also more suitable for *R*-genes with strong effects (Vleeshouwers *et al.* 1999). From this perspective, $R_{Pi\text{-}mcd1}$ should be considered as an *R*-gene with a weak effect. The phenotype of $R_{Pi\text{-}mcd1}$ thus resembles the incompatible reaction of the *S. demissum* differentials *R10* and *R11* (Huang *et al.* 2005), supporting our conclusion that $R_{Pi\text{-}mcd1}$ may belong to the family of NBS-LRR genes.

We have never observed any race-specific interaction for $R_{Pi\text{-}mcd1}$. The 1-2 week delay in infection in the field has been observed consistently in material from various back-cross generations, and irrespective of the late blight races (unpublished results). The material is also used by commercial breeders, who annually confirm the consistent effect of delay of infection against randomly blown-in *P. infestans* isolates. Therefore, $R_{Pi\text{-}mcd1}$ cannot be regarded as a broken *R*-gene (1st model). Neither can $R_{Pi\text{-}mcd1}$ be regarded as a gene involved in residual resistance (3rd model), because from a historical perspective the $R_{Pi\text{-}mcd1}$ locus never conferred complete resistance (was never unbroken), and all races ultimately sporulate (there is no distinction between compatible and incompatible races).

This effect results in the conclusion that $R_{Pi\text{-}mcd1}$ combines two features. It is both a weak resistance gene, as well as a broad spectrum (race non-specific) resistance gene. The value of a weak *R*-gene in economic terms is nevertheless considerable. When infection is delayed for one week in organic potato cultivation, at a biomass production of 1 ton per hectare per day, the economic value of such a delay exceeds €1000,- per hectare.

For decades, it has been advocated that breeding for durable late blight resistance should require the screening of potato germplasm for the absence of *R*-genes. These non-durable *R*-genes only blurred the view on the level of partial, race-non-specific, field resistance. To our opinion this is a flawed strategy and may have unnecessarily removed *R*-genes from the potato gene pool. Moreover, it ignores the possible residual effect of broken *R*-genes, as was demonstrated by Stewart *et al*. (2003). The $R_{Pi\text{-}mcd1}$ gene described in this paper indicates that partial resistance could well be *R*-gene based. We conclude that NBS-LRR-genes, sometimes in black and white, but also in many shades of gray, all posses value in breeding for late blight resistance.

This example of $R_{Pi\text{-}mcd1}$ illustrates the unwarranted relation between phenotype, experimental approach and scientific model. Non-absolute phenotypic differences should not necessarily be addressed by a quantitative genetic approach. Quantitative genetic studies should not necessarily result in QTLs and exclude *R*-genes from the hypothesis tested. In our opinion, the distinction that *R*-gene based resistance should always give absolute resistance and partial resistance is always mediated by QTLs should be re-evaluated.

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Chapter 3

The effect of pyramiding *Phytophthora infestans* resistance genes $R_{Pi\text{-}mcd1}$ and $R_{Pi\text{-}ber}$ in potato

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Abstract

Despite efforts to control late blight in potatoes by introducing *R*-genes from wild species into cultivated potato, there are still concerns regarding the durability and level of resistance. Pyramiding *R*-genes can be a solution to increase both durability and level of resistance. In this study, two resistance genes, $R_{Pi\text{-}mcd1}$ and $R_{Pi\text{-}ber}$, introgressed from the wild tuber bearing potato species *Solanum microdontum* and *S. berthaultii* were combined in a segregating diploid *S. tuberosum* population. Individual genotypes from this segregation population were classified into four groups, carrying no *R*-gene, with only $R_{Pi\text{-}mcd1}$, with only $R_{Pi\text{-}ber}$, and a group with the pyramided $R_{Pi\text{-}mcd1}$ and $R_{Pi\text{-}ber}$ by means of flanking molecular markers. The levels of resistance between the groups were compared in a field experiment in 2007. The group with $R_{Pi\text{-}mcd1}$ showed a significant delay to reach 50% infection of the leaf area of three days. The group with $R_{Pi\text{-}ber}$ showed a delay of three weeks. The resistance level in the pyramid group suggested an additive effect of $R_{Pi\text{-}mcd1}$ with $R_{Pi\text{-}ber}$. This suggests that potato breeding can benefit from combining individual *R*-genes, irrespective of the weak effect of $R_{Pi\text{-}mcd1}$ or the strong effect of $R_{Pi\text{-}ber}$.

Additional keywords: late blight, potato, *R*-gene, *Solanum microdontum*, *Solanum berthaultii*, stacking.

Introduction

Late blight, caused by *Phytophthora infestans*, is a ruthless disease causing great losses in potato yield. One of the solutions has been the use of chemicals. The International Potato Centre (CIP) estimates that about 1 billion dollar per year is spent on fungicides to control late blight in the US, Europe and developing countries (http://gilb.cip.cgiar.org/what-is-late-blight/economic-impact/social-impact-and-economic-importance-of-late-blight/). The loss in yield is nevertheless still considerable, and in addition, the environment suffers from the use of fungicides. In the 1900's, the first successful breeding efforts were undertaken to raise resistance levels of potato varieties by hybridization with wild potatoes (Salaman 1910). Eleven *R*-genes (*R1-R11*), introgressed from *S. demissum*, were the first ones to be used in

potato cultivars. But unfortunately soon after the introduction of many potato cultivars with *S. demissum R*-genes in the 1950's and 1960's, the resistance proved not to be durable. For a period of time, the prevailing strategy to achieve a durable solution against *P. infestans* was thought to be the use of horizontal (race-non-specific) resistance instead of *R*-genes in potato breeding (Colon *et al.* 1995; Landeo *et al.* 1995; Turkensteen 1993; Chapter 2). Potato genotypes showing a hypersensitive response to *Phytophthora* races were discarded, because the non-durable *R*-genes would only blur the view on the level of horizontal resistance. Breeding for late blight resistance would require "*R*-gene free" germplasm (Turkensteen 1993). This strategy has not provided any improvement , mostly because of the inevitable correlation between resistance and late maturity (Simko 2002). Furthermore, it might be a valid assumption that residual effects of defeated *R*-genes could be a factor in horizontal resistance (Stewart *et al.* 2003; Chapter 2). These days the focus is back on *R*-gene mediated resistance (Allefs *et al*. 2005). Since the discovery of the *S. demissum R*-genes, a wide range of wild *Solanum* species have been identified as potential sources for more *R*-genes, e.g. species in the Dutch-German potato collection (Ross and Baerecke 1951; Van Soest *et al.* 1984). Eventually, the resistance genes $R_{Pi\text{-}ber}$ from *S. berthaultii* (Ewing *et al*. 2000), $R_{Pi\text{-}mcd1}$ from *S. microdontum* (Chapter 2) and four *R*-genes from *S. bulbocastanum*: *RB*/$R_{Pi\text{-}blb1,}$ $R_{Pi\text{-}blb2}$, $R_{Pi\text{-}blb3}$ and $R_{Pi\text{-}abpt}$ (Park *et al.* 2005a; Park *et al.* 2005b; Song *et al.* 2003; van der Vossen *et al.* 2003; van der Vossen *et al.* 2005) have been characterised. Currently, these *R*-genes are being deployed in breeding programs and one ($R_{Pi\text{-}blb2}$) is present in the commercial potato varieties "Toluca" and "Bionica" after more than 40 years of traditional crossing and breeding efforts (Hermsen 1966). However, not all of the *R*-genes render absolute levels of resistance in the practical field situation. Worse is the expected durability of these newly identified *R*-genes, as there is no reason to assume a longer durability of the new *R*-genes than of *R1-R11*, especially since these new *R*-genes have to oppose faster evolution rates of *P. infestans* with the introduction of the A2 mating type in the 1980's.

Pyramiding (major) *R*-genes can be one solution to improve on both durability and level of resistance (Nelson 1972). Pyramiding is the accumulation of (*R*)-genes into a single genotype or cultivar and can be achieved using major *R*-genes, defeated *R*-genes, different alleles of one gene, or the same alleles (allele-dosage). Although the potato cultivars such as Pentland Dell (1961) and Escort (1982) demonstrate that

breeders were involved in pyramiding a long time ago, a surprisingly limited number of scientific reports have been published quantifying the effects of pyramided *R*-genes in different plant-pathosystems. Barloy *et al*. (2007) showed a higher level of resistance against cereal cyst nematodes in wheat when pyramiding resistance genes *CreX* and *CreY*. Several groups have reported on the pyramiding of bacterial blight resistance genes in rice and observed higher resistance levels and obtained additionally, a broader spectrum of resistance (Huang *et al.* 1997; Singh *et al.* 2001; Yoshimura *et al.* 1995; Zhang *et al*. 2006). When pyramiding multiple QTLs involved in resistance against barley stripe rust, Richardson *et al*. (2006) reported higher levels of resistance as achieved with individual QTLs. Whereas most studies show an additive effect of pyramiding two *R*-genes, the study of Sharma *et al*. (2004) did not. Marker-assisted pyramiding of the brown planthopper resistance genes *Bph1* and *Bph2* on rice chromosome *12* resulted in a resistance level of the pyramided line equivalent to that of the *Bph1*-single introgression line.

Slightly different are the examples of studies on the effect of allele-dosage. Toxopeus (1957) observed no additional *P. infestans* resistance in duplex R_3 or triplex R_3 genotypes compared to simplex R_3 genotypes. Likewise, no effect of allele dosage was observed in a study with H_1, an *R*-gene against *Globodera rostochiensis* in potato, where numbers of developed cysts were equal on genotypes containing simplex or multiplex copies of the H_1 gene (Brodie and Plaisted 1992).

In this paper we describe a potato field trial in which the effect of pyramiding two *P. infestans R*-genes on late blight resistance is studied. The two *R*-genes $R_{Pi\text{-}ber}$ (chromosome *10*) and $R_{Pi\text{-}mcd1}$ (chromosome *4*) used for pyramiding have been described in literature. $R_{Pi\text{-}mcd1}$ is described in Chapter 2. $R_{Pi\text{-}ber}$, originating from *S. berthaultii*, was reported as a new *R*-gene by Ewing *et al*. (2000) and Rauscher *et al*. (2006). According to information on internet by Dr. J. Jones, there are two *R*-genes located on chromosome *10* of *S. berthaultii* named $R_{Pi\text{-}ber1}$ and $R_{Pi\text{-}ber2}$ (http://www.ayeaye.tsl.ac.uk/index.php?option=com_content&task=view&id=98&Itemid=59). They are both roughly in the same position as the previously identified $R_{Pi\text{-}ber}$ (Ewing *et al.* 2000; Rauscher *et al.* 2006). $R_{Pi\text{-}ber1}$ may be the same gene as $R_{Pi\text{-}ber}$. Because we were uncertain if the *S. berthaultii* resistance from our accession CGN 17823 / PI265858) was the same locus as the previously identified $R_{Pi\text{-}ber}$, we confirmed its map position using data from a field experiment. The data of this field

experiment also confirmed that the resistance conferred by $R_{Pi\text{-}ber}$ is not absolute, but can be described as strong in effect. To study the effect of pyramiding, it is necessary that both used *R*-genes do not give absolute resistance. From our own experience with $R_{Pi\text{-}mcd1}$, an *R*-gene originating from *S. microdontum*, we knew that this *R*-gene gives a weak resistance to *P. infestans* in a field trial (Chapter 2). Since both *R*-genes don't give absolute resistance, we deemed them suitable for studying putative quantitative differences in resistance level when pyramiding these *R*-genes.

We developed a pyramiding population by crossing two diploid parents that were both heterozygous for one of the *R*-genes. In theory, the full-sib offspring can be divided into four groups, carrying no *R*-gene, with only $R_{Pi\text{-}mcd1}$, with only $R_{Pi\text{-}ber}$, and a group with the pyramided $R_{Pi\text{-}mcd1}$ and $R_{Pi\text{-}ber}$. The advantage of making one pyramiding population instead of comparing cultivars with different and combined *R*-genes, is that the genetic background of the groups in one population is on average the same. The four groups cannot be differentiated by late blight assays, unless the different resistance spectra (if any) of the *R*-genes can be used. However, because the genetic position of both *R*-genes $R_{Pi\text{-}ber}$ and $R_{Pi\text{-}mcd1}$ are known, we could make use of molecular markers flanking the *R*-genes. These molecular markers proved an accurate tool to genotype the pyramiding population and assign the genotypes into the four groups.

Materials & Methods

Plant material and *P. infestans* resistance assays

Two segregating diploid populations were developed. The first population RH97-739 (see Figure 1) was developed to verify the map position of $R_{Pi\text{-}ber}$ and to develop flanking DNA markers. The second population RH03-424 (see Figure 2) was developed to identify four groups of descendants without, with either or both *R*-genes.

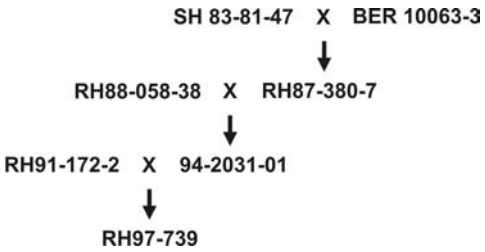

Figure 1: Pedigree of $R_{Pi\text{-}ber}$ mapping population RH97-739 used to develop flanking markers.

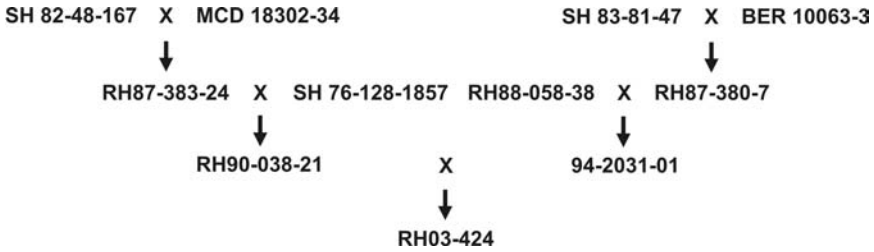

Figure 2: Pedigree of pyramiding population RH03-424 which combines none, either or both the *R*-genes from *Solanum microdontum* and *S. berthaultii*.

The first population, which was used to characterise the $R_{Pi\text{-}ber}$ gene used in this experiment was derived from *S. berthaultii* gene bank accession CGN 17823 / PI265858. This gene might differ from the *R*-gene from the *S. berthaultii* gene bank accession PI473331 (Ewing *et al*., 2000 and Rauscher *et al*., 2006). To verify that the position of both *R*-genes ($R_{Pi\text{-}ber1}$ and $R_{Pi\text{-}ber}$) is identical, a disease assay was performed with the interspecific mapping population RH97-739 (Figure 1). Eighty-eight genotypes were tested in a field experiment in 2005, as well as in a detached leaf assay, whereas the population size for marker analysis was 79 descendants. The field assay was performed *in duplo*, in a randomised complete block design. Inoculation took place with a spore suspension of a complex *P. infestans* isolate IPO-82001 (race 1,2,3,4,5,6,7,10,11) as described by Vleeshouwers *et al*. (1999). Infection was scored 18 and 25 days after inoculation. The genotypes could be scored in a qualitative fashion and were classified as either resistant when infection was $\leq$ 50% of the average leaf area or susceptible when $\geq$ 95% of the average leaf area was infected. The detached leaf assay was performed with leaves collected from greenhouse plants and were tested *in duplo* as described by Vleeshouwers *et al*. (1999). The cultivar Bintje and parents RH91-172-2 and 94-2031-01 were included as control plants.

Complex isolates IPO-82001 (race 1,2,3,4,5,6,7,10,11) and IPO-90128 (race 1,3,4,6,7,8,10,11) were used to determine leaf resistance. Inoculation was performed as described in Vleeshouwers *et al*. (1999) except that the concentration of the inoculum was adjusted to 5 x 10^5 zoospores/ml. In case of discrepancy between field and detached leaf assay results the field data were deemed most reliable.

The second population, also referred to as the pyramiding population RH03-424 (n=93) descended from a cross between $R_{Pi\text{-}mcd1}$ donor RH90-038-21, a BC1 of *S. microdontum* accession BGRC 18302 (CGN21342) and $R_{Pi\text{-}ber}$ donor 94-2031-01, a BC1 of *S. berthaultii* accession BGRC 10063 (CGN 17823 / PI265858). The pedigree of RH03-424 is presented in Figure 2. The resistance gene $R_{Pi\text{-}mcd1}$ is located on the short arm of chromosome *4* and gives a delay of infection to *P. infestans*. For more details concerning the resistance gene $R_{Pi\text{-}mcd1}$, we would like to refer to Chapter 2.

In 2007 a field assay was performed on 93 individuals of pyramiding population RH03-424. The location of the field was the "Hoge Born", in the area near Wageningen, NL on sandy soil. Experimental design was a randomised complete block design, in two replications. Per replication, 4 tubers per genotype were planted. Astarte, Bildtstar and Eersteling were used as standards. Tubers were planted at the 17th of April. Inoculation took place on the 26th of June with a spore suspension of a complex *P. infestans* isolate IPO-82001 (race 1,2,3,4,5,6,7,10,11) as described by Vleeshouwers *et al*. (1999). Foliage infection levels were scored 15 times during 6.5 weeks. The percentage of diseased leaf area was recorded on a scale comprising 16 classes, corresponding to 0, 1, 2, 5, 10, 20, …, 80, 90, 95, 99, 100 percent diseased leaf tissue. The four plants per genotype were scored as one experimental unit.

Maturity type was observed on a fungicide protected field experiment, on clay soil, in August of 2006 and 2007. Maturity type was scored, approximately 130 days after planting on an ordinal scale ranging from 2 (green and vigorous; late to very late) to 6 (yellowing and plant is sagging) to 9 (dead; very early plant types). Four plants per genotype were treated as one experimental unit. Observations of 2006 and 2007 were averaged.

Maturity Corrected Resistance (MCR) was obtained essentially as described by Visker *et al*. (2003), where MCR is estimated from the initial resistance value using

maturity as co-variate. MCR thus represents the difference between the observed resistance value and the resistance value as expected given its maturity. When resistance and maturity data from this population or from potato cultivars from the Dutch National List are plotted in a graph, the regression slope of their correlation is approximately -1. This means that one unit gain on the maturity scale is decreasing late blight resistance with one unit (Visker 2005). Therefore, MCR values (or residuals) can be obtained easily by calculating the proportion of leaf area infected minus maturity value, where maturity was adjusted to the late blight scale (0-100) by multiplication with a factor 10. To display MCR values in a graph along with uncorrected resistance, the MCR values were increased with a constant value that equals the average maturity of the population. (MCR = foliage infection – (maturity value x 10) + (average maturity x 10)).

Genomic DNA extraction

Genomic DNA was isolated from young leaf material, harvested from young greenhouse grown plants, as described by van der Beek *et al*. (1992). Fresh tissue was ground at room temperature in STE-extraction buffer with steel balls using a Retsch machine (Retsch Inc., Haan, Germany). All volumes of the DNA extraction procedure were adapted to 2 ml deep 96-wells Costar plates (Corning Inc., Corning, NY, U.S.A.).

DNA marker assays

The verification and mapping of the $R_{Pi\text{-}ber}$ gene was performed with CAPS markers CT214 (Rauscher *et al.* 2006) and TG63 and SCAR marker Q133. TG63F2/R2 primers were newly developed on TG63 DNA sequence of a genotype of *S. berthaultii* accession BGRC 10063 (CGN 17823 / PI265858) amplified with TG63F1/R primers (Rauscher *et al.* 2006). Marker Q133, was newly developed using DNA sequence information (Genbank AF404451) of a Resistance Gene Analogue which maps to chromosome *10* of tomato (Pan *et al.* 2001). Genotyping of the resistance locus $R_{Pi\text{-}mcd1}$ was performed with chromosome *4* specific CAPS marker TG339 (Chapter 2). All primers were developed with Primer3 software (Rozen and Skaletsky 2000). Primers and PCR conditions are listed in Table 1. Subsequently, the flanking molecular markers were tested for usability on the parents of the pyramiding population because it might be possible that polymorphisms found in one population can not be used in other populations. However, all markers were usable for

genotyping RH03-424 with the restriction enzymes identified in the $R_{Pi\text{-}mcd1}$ or $R_{Pi\text{-}ber}$ mapping population. The molecular markers allowed the pyramiding population to be divided into 4 groups: (1) without *R*-genes, (2) with $R_{Pi\text{-}mcd1}$ present, (3) with $R_{Pi\text{-}ber}$ present, and (4) containing both *R*-genes $R_{Pi\text{-}mcd1}$+ $R_{Pi\text{-}ber}$ pyramided.

Table 1: Overview of the markers, primers and PCR conditions used in this study.

Marker	Primersequences 5'→3'	Annealing temperature and extension time	RE Digestion
CT214	F: AACGCGAAAGAGTGCTGATAG R2: CCCGCTGCCTATGGAGAG T	Tm 60 ºC, 60 sec	*Dde*I
TG63	F2: TCCAATTGCCAGACGAA R2: GAGAAGGCCCTTGTAAGTTT	Tm 55 ºC, 75 sec	*Bme*1390I
TG339	F:GCTGAACGCTATGAGGAGATG R:TGAGGTTATCACGCAGAAGTTG	Tm 56 ºC, 90 sec	*Mnl*I
Q133	F: TCATCTCCTCAAAGAATCAAG R2: ATCTCCCCATTGACAACCAA	Tm 50 ºC, 30 sec	

Linkage map construction of $R_{Pi\text{-}ber}$

The marker order of chromosome *10* specific markers Q133, TG63, CT214 including $R_{Pi\text{-}ber}$ was determined by RECORD (van Os *et al.* 2005a) and visually inspected by graphical genotyping the raw data in MS-Excel using the conditional formatting of cells conditional to marker genotype and linkage phase. Map distances were calculated based on the frequency of recombination between the markers, and in view of the short distances no adjustment for putative double cross-overs was made.

Statistical analysis

Analyses were performed with MS-Excel and comprised the Chi-square test for goodness-of-fit and the analysis of group differences in resistance level in the pyramiding population using a 2-sided *t*-test assuming unequal variances.

Results

Mapping of $R_{Pi\text{-}ber}$

The response of the genotypes of the $R_{Pi\text{-}ber}$ mapping population RH97-793 in the field assay could be scored in a discrete fashion, where 49 genotypes could be scored as resistant (≤ 50% infection of average leaf area) and 37 as susceptible (≥ 95% infection of average leaf area). Two genotypes could not be scored unambiguously. The detached leaf assay showed the same segregation pattern as compared with the field assay, except for 4 differences, probably misclassifications due to mildew infection in the detached leaf assay. Therefore, subsequent analysis have been based on results of the field assay. Chromosome *10* specific marker loci Q133, CT214 and TG63 are closely linked with the locus for resistance, at distances of 6.4 cM, 5.1 cM and 1.3 cM respectively, north of $R_{Pi\text{-}ber}$. The order and distances of the markers towards $R_{Pi\text{-}ber}$ was determined by 5 recombinants, and one singleton marker observation.

One of the 5 recombinants suggested the position of TG63 to be located north of $R_{Pi\text{-}ber}$. While taking this marker order, another descendant, with an ambiguous resistance phenotype then resulted in a singleton. Only the alternative marker order with TG63 south of $R_{Pi\text{-}ber}$ would remove this friction, but would cause map friction in the recombinant with an unambiguous late blight phenotype. Therefore the marker order as shown in Figure 3 is the most plausible order as judged from the marker data.

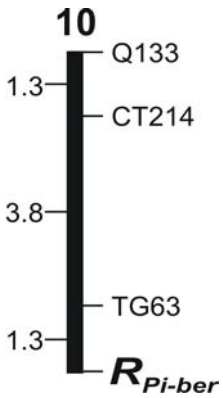

Figure 3: Genetic map of part of chromosome *10* of paternal resistant parent 94-2031-01 including the location of $R_{Pi\text{-}ber}$.

Analysis of the pyramiding population RH03-424 with markers flanking $R_{Pi\text{-}mcd1}$ and $R_{Pi\text{-}ber}$

To identify which of either resistance genes are present in each offspring of the pyramiding population (n=93), molecular markers flanking the *R*-genes were used. The pyramiding population RH03-424 could be differentiated into 4 groups: (1) without *R*-genes, (2) with $R_{Pi\text{-}mcd1}$ present, (3) with $R_{Pi\text{-}ber}$ present, and (4) containing both *R*-genes $R_{Pi\text{-}mcd1}$+ $R_{Pi\text{-}ber}$ pyramided (Table 2). The observed segregation pattern into the four groups deviated significantly from the expected 1:1:1:1 ratio (χ^2 = 16.7; p<0.001). This deviation is largely due to an overrepresentation of resistant genotypes having the $R_{Pi\text{-}ber}$ allele (63:30), whereas the $R_{Pi\text{-}mcd1}$ locus hardly deviates from a 1:1 segregating ratio (56:37 (χ^2 = 3.88; p=0.05).

Table 2: Observed segregation of the descendants of the pyramiding population RH03-424 into four groups; without *R*-genes, with $R_{Pi\text{-}mcd1}$ present, with $R_{Pi\text{-}ber}$ present, and containing both *R*-genes $R_{Pi\text{-}mcd1}$+ $R_{Pi\text{-}ber}$ according to the flanking molecular markers on chromosome *4* and *10*.

	+ $R_{Pi\text{-}ber}$	- $R_{Pi\text{-}ber}$
+ $R_{Pi\text{-}mcd1}$	37	19
- $R_{Pi\text{-}mcd1}$	26	11

Evaluation of the resistance level in the offspring of the pyramiding population

The results described below will first address the effect of the individual genes (a) $R_{Pi\text{-}mcd1}$ and (b) $R_{Pi\text{-}ber}$, respectively, (c) their interaction and (d) the effects after correction for maturity type.

(a) *R*-gene free group vs. $R_{Pi\text{-}mcd}$ group:

One week after inoculation, the *R*-gene free group had an average foliage infection of 66% (Figure 4). The group with descendants with the $R_{Pi\text{-}mcd1}$ allele showed infection as well, but to a lesser degree with 46% infection at this time point. The difference between the groups with and without the $R_{Pi\text{-}mcd1}$ allele was significant from 7 dpi until 14 dpi (2-sided t-test, df = 24-27, p<0.05), with the largest effect at 9 dpi (p = 0.0027). The difference was no longer significant at 17 days post infection (dpi) and later time points, when both groups with or without $R_{Pi\text{-}mcd1}$ approached 100% infection.

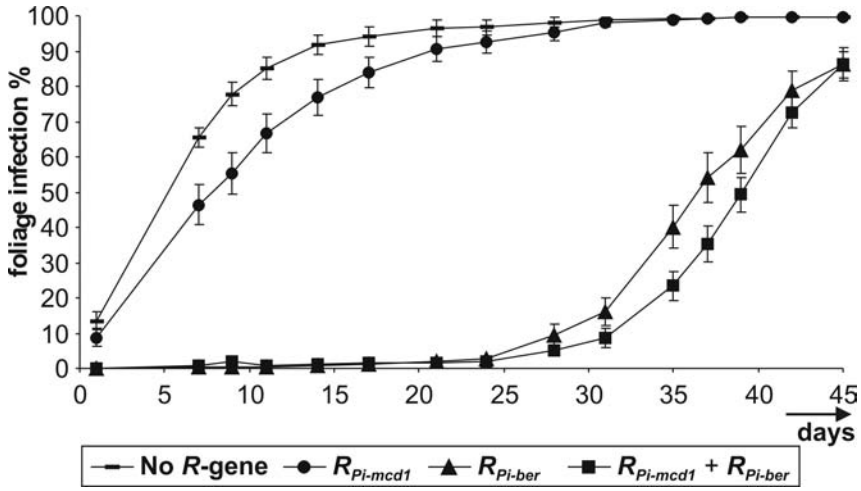

Figure 4: Late blight disease progress curve in proportion of leaf area infected across 45 days post infection (dpi) of the four groups of pyramiding population RH03-424; without *R*-genes, with $R_{Pi\text{-}mcd1}$ present, with $R_{Pi\text{-}ber}$ present, and containing both *R*-genes $R_{Pi\text{-}mcd1}$+ $R_{Pi\text{-}ber}$. Error bars indicate the standard error of the mean.

(b) Resistance level of $R_{Pi\text{-}ber}$

The difference in infection level between the groups with or without the $R_{Pi\text{-}ber}$ allele is much larger compared to the effect caused by the $R_{Pi\text{-}mcd1}$ allele (Figure 4). When the foliage of plants from the *R*-gene free group is more than 90 % infected, the $R_{Pi\text{-}ber}$ bearing groups remain almost free from *P. infestans* symptoms, with an average degree of infection below 5%. This absence of *P. infestans* infection as the effect of the $R_{Pi\text{-}ber}$ allele, remains until 24 dpi, a full 3 weeks longer than the *R*-gene free group, as well as the $R_{Pi\text{-}mcd1}$ group. $R_{Pi\text{-}ber}$ does not provide absolute resistance however. At 24 dpi these plants also start to show late blight infection. In this experiment, the genotypes with $R_{Pi\text{-}ber}$ passed the level of 50% infection on average at 36 dpi, six times later than the *R*-gene free genotypes and 4.5 times later than genotypes with $R_{Pi\text{-}mcd1}$.

(c) Effect of pyramiding $R_{Pi\text{-}mcd1}$ + $R_{Pi\text{-}ber}$

From the first observation date, the presence of the $R_{Pi\text{-}ber}$ allele has a more noticeable effect as compared to the presence of the $R_{Pi\text{-}mcd1}$ allele. The effect of pyramiding of the two resistance genes can only be observed when the plants having the $R_{Pi\text{-}ber}$ allele (with or without $R_{Pi\text{-}mcd1}$) also show infection. A significant contribution (2-sided t-test, df = 45-48, $p<0.05$) of the $R_{Pi\text{-}mcd1}$ allele in plants with $R_{Pi\text{-}ber}$ is observed from 35 dpi and remains significant till 37 dpi, with the largest effect observed at day 35 ($p=0.026$) (Figure 4). Considering that the genetic background of the $R_{Pi\text{-}ber}$ group and the pyramided groups are on average the same, we conclude that the difference in resistance level is due to the additive effect of the $R_{Pi\text{-}mcd1}$ allele. The magnitude of this effect is comparable with the difference observed between the *R*-gene free group and the $R_{Pi\text{-}mcd1}$ group: 50% infection is achieved 3 days later in the pyramided group than in the group containing only the $R_{Pi\text{-}ber}$ allele.

(d) The effect of plant maturity on late blight infection

Within each group there is considerable variation in the level of infection per genotype, where some genotypes containing the $R_{Pi\text{-}ber}$ allele may have a level of infection which is lower than some genotypes in the pyramided group. The infection of both the $R_{Pi\text{-}ber}$ groups begins at 24 dpi, which is 3 months after planting, long enough for first early cultivars to end their life cycle. Maturity types in the pyramiding population varied between 4 and 9 with a mean of 6.9 on a total scale of 1 to 9. Differences in maturity type could potentially influence the level of late blight resistance. The strong correlation between *P. infestans* foliage resistance and late plant maturity resulted in the use of maturity corrected resistance (MCR) (Bormann *et al.* 2004; Visker *et al.* 2004). Therefore, we re-analysed our late blight resistance observations using a maturity corrected dataset, where late blight resistance is compensated for the effect of maturity. MCR was calculated in this study by subtracting maturity values from leaf area infection values, but adding up the average value for maturity of the population, leaving the residuals. This is a simple and valid approach, because analysis of variance components showed that the variance contributed by maturity is of the same magnitude as the variance contributed by the *R*-gene composition.

In Figure 5, the effect is illustrated of this correction for maturity on late blight infection at 35 dpi. When comparing the maturity uncorrected (Figure 5a) vs. the

maturity corrected (Figure 5b) analysis, it becomes apparent that the correction for maturity slightly improves the difference in resistance levels between the $R_{Pi\text{-}ber}$ group and the $R_{Pi\text{-}ber}$ + $R_{Pi\text{-}mcd1}$ group. In addition, the correction for maturity reduced the standard error of the group mean. Removing the heterogeneity in maturity between plants within the four groups thus allowed a more accurate assessment of late blight resistance differences due to *R*-gene composition. The correction for maturity also confirmed that quantitative variation in the observed level of infection was not only caused by *R*-genes but in part caused by maturity. Lastly, the maturity corrected analysis showed that the effect of pyramiding remains statistically significant for a much longer period, from 31 dpi until 39 dpi (9 days) instead of 3 days (2- sided t-test, df = 47-50, $p < 0.05$), with the largest effect at 35 dpi ($p = 0.0014$). In Figure 6, the progress in time of the maturity corrected foliage infection for the four groups is illustrated.

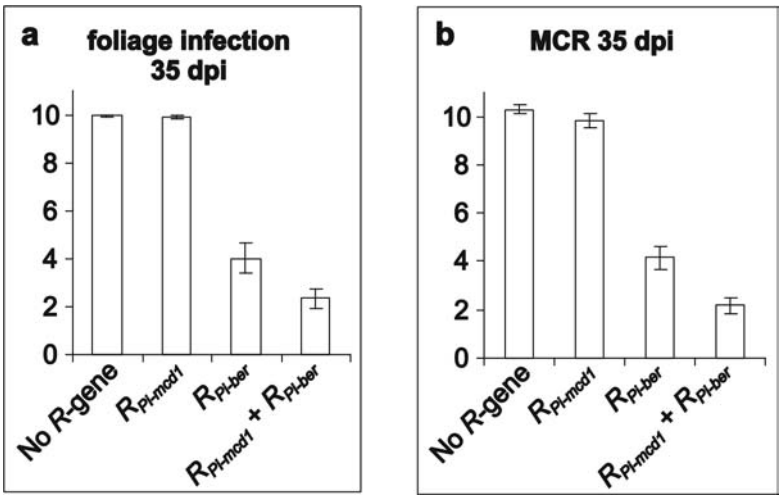

Figure 5: (a) Group averages of foliage infection at 35 dpi. (b) Group averages of maturity corrected resistance (MCR) at 35 dpi. Error bars indicate the standard error of the mean.

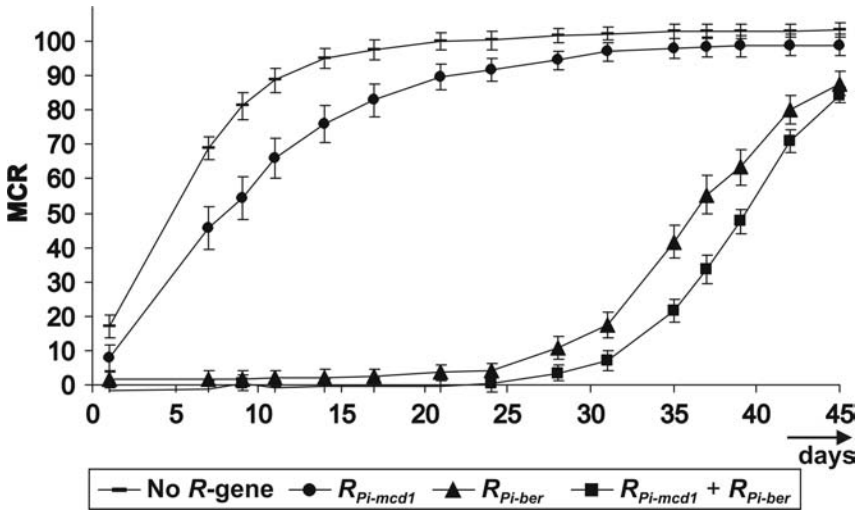

Figure 6: Late blight disease progress curve in proportion of leaf area infected corrected for maturity type across 45 days post infection (dpi) of the four groups of pyramiding population RH03-424; without *R*-genes, with $R_{Pi\text{-}mcd1}$ present, with $R_{Pi\text{-}ber}$ present, and containing both *R*-genes $R_{Pi\text{-}mcd1}$+ $R_{Pi\text{-}ber}$. MCR = maturity corrected resistance. Error bars indicate the standard error of the mean.

Discussion

The individual *R*-genes used in this study have been described before in literature.
The phenotypic effects of resistance based on $R_{Pi\text{-}mcd1}$ are described in Chapter 2. It was then estimated that $R_{Pi\text{-}mcd1}$ causes a delay of infection of about 1-2 weeks. In this experiment though, genotypes with $R_{Pi\text{-}mcd1}$ pass the level of 50% infection on average 3 days later than *R*-gene free genotypes. The shorter delay of infection by $R_{Pi\text{-}mcd1}$ in this experiment is likely caused by the relatively cold and humid weather favouring rapid late blight development in the summer of 2007.

Rauscher *et al*. (2006) mapped $R_{Pi\text{-}ber}$ 5.1 cM south of marker CT214 and 0.6 cM north of marker TG63. The resistance we observed in this study mapped to chromosome *10,* close to marker TG63, but 1.3 cM south of marker TG63. Although the position of marker TG63 relative to $R_{Pi\text{-}ber}$ differs in this study with the marker order shown by Rauscher *et al*., we do not wish to draw strong conclusions on only one recombination event. Therefore we cannot present conclusive evidence that this

resistance is a different locus as the previously reported $R_{Pi\text{-}ber}$. The difference in position of marker TG63 could be attributed to false positive/negative marker or phenotypic scoring which can make a large impact when dealing with a small population and a small number of markers. An alternative explanation could be that marker TG63 is located inside (and thus flanked by) a large cluster of *R*-gene homologues. The use of one accession or another accession with different functional members of the cluster could lead to alternative mapping orders.

The objective of this research was to pyramid two functional *R*-genes originating from wild potato species in a *S. tuberosum* background and to compare the resistance level of those pyramided *R*-genes with the level of the single *R*-genes. We combined $R_{Pi\text{-}mcd1}$ with $R_{Pi\text{-}ber}$. Both *R*-genes confer incomplete resistance to *P. infestans* but greatly differ in their effect. The $R_{Pi\text{-}mcd1}$ gene offers a small but significant reduction of the infection, lasting only for a short period while the $R_{Pi\text{-}ber}$ resistance gene offers a strong effect. It took 36 days until 50 % of the average leaf area was infected in plants that carry the $R_{Pi\text{-}ber}$ resistance gene. In the field trial the plants with the $R_{Pi\text{-}ber}$ resistance gene did not confer immunity during the entire growth cycle. The failure of the resistance by the end of the growing cycle is an annually observed phenomenon, known from other trials and years using this genetic material (data not shown). This is best explained by the physiological ageing of the plant. Pyramiding of $R_{Pi\text{-}mcd1}$ and $R_{Pi\text{-}ber}$ resulted in a higher level of resistance. This is not an obvious conclusion that should have been expected before starting this experiment. In view of the large effect of the $R_{Pi\text{-}ber}$ gene until late in the growing season, and in view of the short delay of infection at the begin of the infection period we could also have expected that the effect of the $R_{Pi\text{-}ber}$ gene would entirely surpass the modest effect of the $R_{Pi\text{-}mcd1}$ gene. The resistance level in the pyramid group showed an additive effect of $R_{Pi\text{-}mcd1}$ with $R_{Pi\text{-}ber}$ without clear evidence for a ‘law of diminishing returns’.

In our experiment we used a large population of full sibs. This sibling structure would on average nullify the interaction between a specific genetic background and the *R*-genes. One example of such an interaction is the influence of maturity type on resistance level, irrespective of *R*-gene composition. This experimental design proved to be effective, because it allowed us to compensate for maturity effects. In our experiment, the magnitude of the effect caused by the individual *R*-genes was slightly obscured by variation in maturity. When resistance levels were corrected for maturity

type, the difference between pyramided and the single $R_{Pi\text{-}ber}$ resistance levels stayed significant for a longer period of time: nine days instead of three days.

The pyramiding of *R*-genes is a valid strategy if the anticipated benefits exceed the breeding efforts. It is debatable if an infection delay of a couple of days is valuable. In our pyramided group, the resistance level of $R_{Pi\text{-}ber}$ was complemented by the resistance level of $R_{Pi\text{-}mcd1}$. $R_{Pi\text{-}mcd1}$ is known to be effective for a limited period. Therefore, improvement of resistance levels would be more economic when using *R*-genes with stronger effects. Irrespective of the complexities of natural infection with late blight, our findings indicate that combining the *R*-genes $R_{Pi\text{-}mcd1}$ and $R_{Pi\text{-}ber}$ in potato is useful. In view of the additivity of the effects of the $R_{Pi\text{-}mcd1}$ and $R_{Pi\text{-}ber}$ genes, and the lack of evidence for the 'law of diminishing returns', we recommend the development and analysis of potato clones with additionally pyramided *R*-genes to investigate the hypothesis that resistance levels comparable to non-host resistance might be feasible (Heath 2001; Jeuken and Lindhout 2002).

More important than economic considerations, the strategy of pyramiding *R*-genes is valued because of epidemiological and evolutionary considerations. Besides raising the level of resistance, pyramiding *R*-genes might contribute to the durability of resistance. The pathogen would need double or multiple mutations to overcome the resistance. The durability of an *R*-gene can be perceived as the evolutionary potential (mutation and recombination) of the plant pathogen to avoid *R*-gene recognition. In that case *R*-gene durability is based on the evolution of effector molecules and the indispensability of their current function. But when discussing durability of single or pyramided *R*-genes, one should be aware that durability can not be predicted in advance, but can only be reflected upon by historical evaluation. To ensure a maximum lifespan of an *R*-gene though, it is important that single-*R*-gene and pyramided plants are not deployed simultaneously. The single-gene plants would in that case provide a "stepping-stone" for pathogens to overcome each of the pyramided resistance genes (Zhao *et al.* 2003).

Cultivars with pyramided *R*-genes can be developed by traditional and time consuming breeding or with genetic modification. Genetic modification is assumed to be a fast way to insert two or more *R*-genes into an existing variety. A huge benefit is that undesirable linkage drag from the wild potato genome, causing for example an

elevated glycoalkaloid content, is prevented. Most of the *R*-genes against *P. infestans* originate from crossable wild potato species. Therefore the GM product should not be considered as transgenic but as cisgenic (Jacobsen and Schouten 2007). As long as public acceptance for genetic modification is lacking in the EU, classical introgression breeding is required. One positive aspect of the use of diploid progenitor lines is the high frequency of gene transfer of these genes to the tetraploid potato via 4X-2X crosses. On average 80% of the heterozygous *R*-gene in diploids will result in tetraploid offspring with a simplex *R*-gene due to First Division Restitution of the unreduced male-gametes (Hermsen 1984). Molecular markers can facilitate the selection of superior descendants by tracing the *R*-genes in the breeding process and removing linkage drag efficiently.

In this paper we have tested the hypothesis whether or not pyramiding is a valid strategy. There was no *a priori* expectation on the added value of the weaker $R_{Pi\text{-}mcd1}$ locus in the presence of the stronger $R_{Pi\text{-}ber}$ locus. Our results suggest that pyramiding can result in an additive effect of the individual genes on the level of resistance. In our opinion, potato breeding can benefit from combining individual *R*-genes.

Acknowledgements: We gratefully acknowledge René Alles for technical assistance and Björn d'Hoop for critically reading the manuscript.

Chapter 4

GpaXI$^{l}_{tar}$ originating from *Solanum tarijense* is a major resistance locus to *Globodera pallida* and is localised on chromosome *11* of potato

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Abstract

Resistance to *G. pallida* Rookmaker (Pa3), originating from wild species *S. tarijense* was identified by QTL analysis and can be largely ascribed to one major QTL. *GpaXI*$^{l}_{tar}$ explained 81.3 % of the phenotypic variance in the disease test and mapped to the long arm of chromosome *11*. Another minor QTL explained 5.3 % of the phenotypic variance and mapped to the long arm of chromosome *9*. Clones containing both QTL showed no lower cyst counts than clones with only *GpaXI*$^{l}_{tar}$. After Mendelising the phenotypic data, *GpaXI*$^{l}_{tar}$ could be more precisely mapped near markers GP163 and FEN427 thus anchoring *GpaXI*$^{l}_{tar}$ to a region with a known *R*-gene cluster containing virus and nematode resistance genes.

Key words: Potato cyst nematode, monogenic resistance, potato, *Solanum* species

Introduction

The white potato cyst nematode (PCN) *Globodera pallida* (Stone) is a major pest in potato. Control of PCN takes place with nematicides, crop rotation and (partially) resistant cultivars (Trudgill *et al.* 2003). The cultivated potato, *Solanum tuberosum* ssp. *tuberosum*, is not resistant to *Globodera* species. Wild species of potato have been used over the last decades to transfer resistance into potato cultivars. Early work on resistance breeding has been concentrated on the yellow potato cyst nematode *G. rostochiensis*, although the distinction between *G. rostochiensis* and *G. pallida* has been introduced only since 1974 (Stone 1972). Breeders succeeded in producing potato cultivars resistant to *G. rostochiensis*, predominantly by incorporating the *H1* gene from *S. tuberosum* ssp. *andigena* CPC 1673 (Ellenby 1952; Huijsman 1957; Ross 1979). Since the discovery of *H1*, many more resistance genes against *G. rostochiensis* have been identified (reviewed by Gebhardt and Valkonen 2001). Due to the extensive use of *G. rostochiensis* resistant cultivars, *G. pallida* populations, free of competition from *G. rostochiensis*, have become the majority of the cyst nematode pest population in Europe. Great emphasis is now placed on producing varieties with resistance against a broad spectrum of populations (Hancock 1996; Turner 1989).

While breeders succeeded in producing potato varieties resistant to *G. rostochiensis*, breeding for resistance to *G. pallida* was initially hampered by the seemingly quantitative inheritance of the resistances first identified in *S. vernei* and *S. tuberosum* spp. *andigena* (Dale and Phillips 1982; Ross 1986). The resistance to pathotypes Pa2 and Pa3 from *S. tuberosum* ssp. *andigena* CPC 2802 was at first thought to be monogenic (the *H3* gene) (Howard *et al.* 1970), but was later proven to be polygenic (Dale and Phillips 1982). The higher diversity of the *G. pallida* populations compared to *G. rostochiensis* populations also hampered incorporation of effective resistance (Folkertsma 1994; Schnick *et al.* 1990).

Monogenic resistance to *G. pallida* has been identified, some appear to be only effective against specific *G. pallida* populations, such as the *H2* locus from *S. multidissectum*, conferring resistance to *G. pallida* populations of pathotype Pa1 (Dunnett 1961), and the *Gpa2* gene. *Gpa2*, derived from *S. tuberosum* spp. *andigena* CPC 1673 confers resistance to a small, distinct population of *G. pallida* Pa2. *Gpa2* maps on chromosome *12* of potato in a single cluster of virus and nematode resistance genes and has been cloned. The protein encoded by *Gpa2* turned out to be of the class of LZ-NBS-LRR type plant resistance genes (Arntzen *et al.* 1994; Rouppe van der Voort *et al.* 1999; van der Vossen *et al.* 2000). Although broad spectrum resistance to *G. pallida* has been thought to be based on polygenic inheritance, evidence indicates that major genes can be involved in broad spectrum resistance as well. Kreike *et al.* (1994) reported that the *Gpa* locus derived from *S. spegazinnii* conferred resistance to at least two distinct *G. pallida* populations. This major locus on chromosome *5* explained about 50% of the total variance for resistance to both pathotypes Pa2 and Pa3. Rouppe van der Voort *et al.* (2000) identified two QTL that are likely derived from *S. vernei*. One of the two loci, *Gpa5*, explained 61% of the total variation and the resistance conferred by this locus appears to work against a range of *Globodera* populations. The other locus, *Gpa6*, explained 24 % of the total variation and acts in a pathotype specific way. Interestingly, both QTL combined give additive resistance levels. The same holds true for potato clones carrying both QTL $GpaV^{s}_{spl}$ and $GpaXI^{s}_{spl}$ (Caromel *et al.* 2005). These *S. sparsipilum* derived QTL were mapped on chromosome *5* and *11* respectively. When combined they do not only show an additive effect on sex ratio of *G. pallida*, but also give a necrotic reaction in roots infected by nematodes; an effect not seen with the individual QTL. Another locus, *Grp1*, with resistance against both PCN species (Rouppe van der Voort *et al.* 1998a)

showed resistance to *G. rostochiensis* line Ro_5-22 and *G. pallida* populations Pa_2-D383 and Pa_3-Rookmaker. *Grp1* was mapped on chromosome *5* in the same region as *Gpa* and *Gpa5*. However, it has not been described in literature that any of the wild *Solanum* sources used to introgress *Grp1* showed resistance against both *G. rostochiensis* and *G. pallida*. It is not excluded that the combined resistance against *G. rostochiensis* and *G. pallida* of *Grp1* is caused by two, or possibly three, tightly linked *R*-genes.

For most *G. pallida* resistance loci described above, the phenotypic segregation data indicated quantitative inheritance. DNA markers offered the potential to trace the Mendelian loci underlying quantitatively inherited nematode resistance and in many cases the resistance appeared to be controlled by single major genes explaining a large proportion of the phenotypic variance. Moreover, the fact that most PCN resistances have been mapped in well known resistance clusters indicates the likelihood that these major loci are NBS-LRR genes and operate on a gene-for-gene basis (Gebhardt and Valkonen 2001).

In this study, we report a major-effect locus involved in resistance against *G. pallida* Pa3-Rookmaker originating from *S. tarijense*. Qualitative interpretation of the resistance data allowed mapping of the major-effect locus to a known cluster of resistance genes on the long arm of chromosome *11*.

Materials and methods

Plant material

The diploid BC1 mapping population RHAM-061 was obtained from a cross of resistant parent RH90-011-4 and susceptible *S. tuberosum* parent RH89-039-16, as shown in Figure 1. RH90-011-4 was obtained from an interspecific cross between *S. tarijense* (BGRC 24717 / CGN 18107) and MON-46, a dihaploid clone of the susceptible cultivar Mondial.

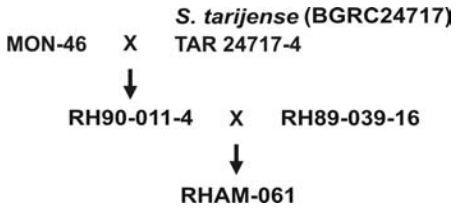


Figure 1: Pedigree of the interspecific $GpaXI^{l}_{tar}$ mapping population RHAM-061.

Nematode resistance assays

A closed container test (Phillips *et al.* 1980) with *G. rostochiensis* populations ASCRI (Ro1), C262 (Ro2_3), G 1510 (Ro5) was performed on the *S. tarijense* parent RH90-011-4. After 8 weeks in the dark at 18 ºC, the newly formed cysts that were visible from the outside were counted.

All further *G. pallida* resistance assays were arranged in randomised complete block designs, including parents. 188 offspring genotypes of RHAM-061 were multiplied *in vitro* and transplanted in 500 ml clay pots to the greenhouse in 3 replicates. Eight replications of the parents as well as the susceptible standard Bintje were included. *G. pallida* population Rookmaker (Pa3), originally sampled from a heavily infested site in Valthe, the Netherlands, was used for the inoculation. Population Rookmaker is one of the most virulent *G. pallida* populations in the Netherlands (Bakker *et al.* 1992). Four weeks after transplanting, the plants were inoculated. Inoculum of nematodes was prepared as described by (Rouppe van der Voort *et al.* 1997a). Cysts were soaked overnight in tap water before crushing to obtain eggs and second-stage juveniles (J_2). The egg/J2 suspension was then sieved through a 100- µm sieve to remove cell walls and debris. The suspension was inoculated to the plants to a final density of 6 eggs/J_2 per ml soil. Two and a half months after inoculation, the cysts were elutriated with a Fenwick can and counted (Fenwick 1940).

DNA extraction

Genomic DNA was isolated from young leaf material as described by van der Beek *et al*. (1992). Tissue was ground with steel balls using a Retsch machine (Retsch Inc., Haan, Germany) in the STE-buffer, with volumes adapted to 2 ml deep 96-wells Costar plates (Corning Inc., Corning, NY, U.S.A.). DNA was visualised on agarose gel to check the integrity.

_Eco_RI/_Mse_I markers

AFLP was performed according to (Vos *et al.* 1995). Fourteen *Eco*RI/*Mse*I primercombinations were used: eAACmCCA, eAACmCCT, eAACmCGA, eAACmCTG, eAAGmCCT, eAAGmCGA, eACAmCAA, eACAmCCA, eACAmCTA, eACTmCAA, eACTmCAG, eAGAmCAG, eAGAmCAT, eAGAmCTG. AFLP fragments were visualised on radioactive gels and scored visually for absence/presence.

PCR markers

Two chromosome *11* specific markers, GP163 (Brigneti *et al.* 1997) and FEN427, were used. CAPS marker GP163 is a previously converted RFLP marker, previously used to map potato virus Y resistance gene Ry_{sto} on chromosome *11* (Brigneti *et al.* 1997). CAPS marker FEN427 was developed on the basis of AFLP marker pATmCAC_427 which was previously used to map $R_{Mc1\text{-}fen}$ on chromosome *11* (Draaistra 2006). AFLP band pATmCAC_427 was excised from gel and dissolved in 50 µl water. Supernatant was re-amplified with P+0 and M+C primers and purified with a G50 column before sequencing. PCR primers for FEN427 were designed on the sequence of AFLP marker pATmCAC_427 with Primer3 software (Rozen and Skaletsky 2000). PCR-products of GP163 and FEN427 were digested with 27 restriction enzymes to identify polymorphic sites (Brugmans *et al.* 2003).

Map construction

The grouping of markers in linkage groups and the marker order was calculated using the software package JoinMap (Stam 1993). Only AFLP markers with LOD scores >3.0 were included in mapping. Initially the maternal and paternal linkage groups were constructed separately, by taking only the 1:1 segregating AFLP loci only (Aa × aa and aa × Aa for the maternal and paternal maps respectively). The paternal linkage groups could be assigned to potato chromosomes using a reference mapping population which has the susceptible parent RH89-039-16 in common. This reference map SHxRH (Rouppe van der Voort *et al.* 1997a; van Os *et al.* 2006) was aligned along with other maps, and resulted in an online catalogue of AFLP markers covering the potato genome (Rouppe van der Voort *et al.* 1998b). Once the chromosome numbers were known of the paternal linkage groups, subsequently the maternal linkage groups could be aligned using the so called ‘bridge markers’. Bridge markers

are a small subset of the segregating AFLP loci, which are heterozygous in both parents (Aa × Aa and thus segregating in a 3:1 ratio).

The maternal linkage group, corresponding to potato chromosome *11* including the locus involved in PCN resistance gene was recalculated with RECORD (van Os *et al.* 2005a), to assess putative ambiguities in marker order. With the information on marker order, the raw data were inspected for spurious data points (singletons). Singletons are easily recognised in graphical genotypes which are generated from the raw data in MS-Excel where cell colour is formatted conditional to marker observation and linkage phase.

Data analysis

QTL analysis of the resistance data was performed with MapQTL 5.0 (van Ooijen and Maliepaard 1996). Data were transformed by taking the natural logarithm of the average cyst number (+1) per genotype, to obtain a uniform distribution of the variance. The heritability was estimated as follows: $h^2 = \sigma^2_g / (\sigma^2_g + \sigma^2_e/n)$, where n is the number of replications, σ^2_e is the expected mean square of the residuals and σ^2_g is retrieved from the ANOVA table as follows: $E(MS)genotypes = n\sigma^2_g + \sigma^2_e$.

Two parametric methods: interval mapping and multiple-QTL mapping (MQM) were applied. The threshold for assigning a QTL was determined by a permutation test, as implemented in MapQTL 5.0.

Classification of cyst counts into resistant / susceptible phenotypes

For the qualitative mapping of the resistance locus, data of the first nematode resistance assay were used. All genotypes up to a maximum mean of 8 newly formed cysts were assigned as resistant and genotypes with at least 25 newly formed cysts were assigned as susceptible.

Results

The inheritance of PCN resistance

In an evaluation of tuber bearing *Solanum* species by The Centre for Genetic Resources (CGN), it was detected that *S. tarijense* accession BGRC 24717 contained resistance against *G. pallida* pathotype Pa3 (http://www.cgn.wur.nl). The inheritance of *G. pallida* resistance was analysed by testing a segregating BC1 population RHAM-061 of *S. tarijense* (BGRC 24717). Plant material inoculated with *G. pallida* population Rookmaker clearly showed a continuous distribution of the average number of cysts developed per genotype, ranging from 1 to 419. The resistant *S. tarijense* F1 parent RH90-011-4 developed on average 2 cysts while the susceptible *S. tuberosum* parent RH89-039-16 developed on average 132 cysts. An example of cysts on a susceptible plant is shown in Figure 2. Fourteen genotypes of RHAM-061 with highly variable numbers of cysts in the different replications were excluded from further analysis. The variance in average number of cysts per genotype greatly differed between resistant and susceptible genotypes. A uniform distribution of the variance was obtained by taking the natural logarithm of the average cyst number per genotype. Analysis of the variance within and between genotypes showed that the genotypes differed significantly ($P<0.001$). Heritability was estimated to be 0.82.

To test if the working spectrum of the *G. pallida* resistance also included *G. rostochiensis* resistance, the *S. tarijense* derived F1 parent RH90-011-4 was inoculated with *G. rostochiensis* in a container test. With all pathotypes however (Ro1, Ro2_3 and Ro5), large numbers of cysts were developed (between 20 and 60 cysts). The resistance originating from *S. tarijense* against *G. pallida* Rookmaker is therefore not also functional against *G. rostochiensis*.

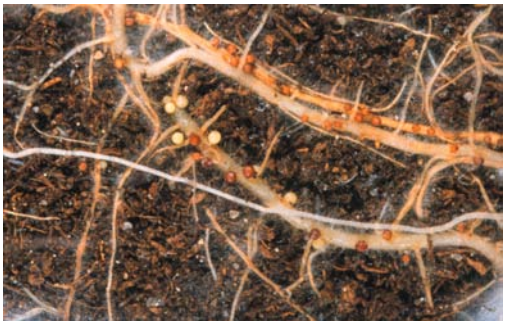


Figure 2: Swollen females (appearing as white cysts) and brown cysts of *G. pallida* formed on roots of a susceptible potato plant.

Map construction

A linkage map was constructed using the segregating markers of 14 *Eco*RI/*Mse*I primer combinations. In total 518 segregating markers were identified. 298 AFLP markers segregated from maternal genotype RH90-011-4, 155 markers from paternal genotype RH89-039-16. There were 65 bridge-markers which are heterozygous in both parents, which were used to connect maternal and paternal linkage groups as identified on the basis of the 1:1 segregating maternal or paternal marker loci. The map of maternal clone RH90-011-4 comprised 12 linkage groups with a total map length of 830.3 cM. The markers of paternal clone RH89-039-16 (RH) were assigned into 12 linkage groups with a total length of 561.1 cM. 19 AFLP markers remained unassigned at LOD=3. Chromosome numbers of RH were identified by aligning the RH linkage groups from this mapping population with the RH linkage groups of reference maps in our lab (Rouppe van der Voort *et al.* 1997a; Rouppe van der Voort *et al.* 1998b; van Os *et al.* 2006). The 65 bridge-markers were sufficiently equally distributed over the 24 linkage groups and allowed to pair all 12 maternal linkage groups to their homologous paternal (RH) linkage groups, hereby also identifying the chromosome numbers and orientations of the maternal linkage groups.

QTL mapping

The distribution of the ln-normalised cyst counts of the disease test of RHAM-061 was clearly bimodal which could be indicative of a large-effect QTL involved in quantitative resistance. QTL analysis was applied on all maternal and paternal linkage groups to identify all possible resistance factors. With the interval mapping method of MapQTL, one large-effect QTL was detected on maternal chromosome *11* (LOD=57), which explained 81.3 % of the phenotypic variance. No other marker -

trait associations exceeded the LOD thresholds of the individual linkage groups as determined by the permutation test. To enhance the power to detect minor-effect QTLs, the MQM mapping method was applied with the QTL on chromosome *11* as a cofactor. This resulted in the identification of a QTL (LOD=4.4) on the long arm of chromosome *9*. The percentage of explained phenotypic variance of this QTL was only 5.3%. Because of the small effect we assume that the resistance is essentially monogenically inherited and located on chromosome *11*.

Qualitative mapping of resistance

In view of the single locus involved in PCN resistance, as identified with QTL analysis, we proceeded with a qualitative genetic approach. The disease test phenotypes were used for a tentative classification of the offspring, where 76 genotypes containing on average less than 8 cysts (ln 8+1 = 2.2) were assigned as resistant and 64 genotypes with an average of more than 25 (ln 25+1 = 3.3) were assigned as susceptible. The remaining 34 genotypes of intermediate phenotype were excluded from further analysis (Figure 3a). The transmission of the resistance from the wild species to the BC1 mapping population, and the observed segregation ratio which is consistent with a 1:1 distribution (χ^2 = 0.067, p = 0.80) allows to conclude that the effects can be explained by a single dominant gene.

The qualitatively segregating resistance data were added to the maternal marker dataset. According to JoinMap analysis, the resistance locus showed linkage with 10 markers on chromosome *11* in the same region where the resistance QTL was previously mapped. The marker order of chromosome *11* including the resistance locus was verified with RECORD. The resistance locus, which we propose to name *GpaXI*$^{l}_{tar}$, is localised on a distal position on the long arm of chromosome *11* between AFLP markers eACTmCAA_174 and eAGAmCTG_222.

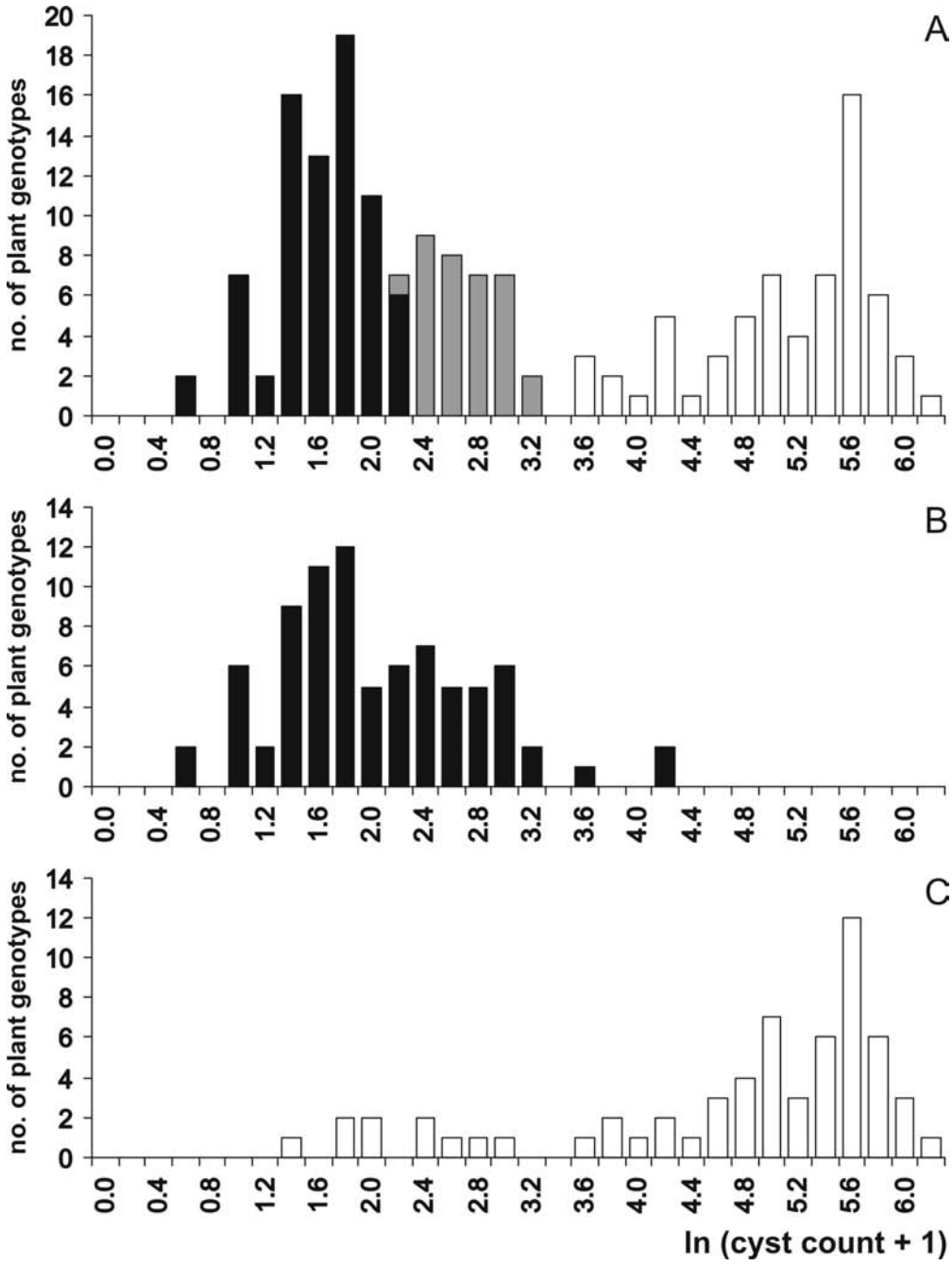

A
B
C
no. of plant genotypes
ln (cyst count + 1)
0.0
0.4
0.8
1.2
1.6
2.0
2.4
2.8
3.2
3.6
4.0
4.4
4.8
5.2
5.6
6.0

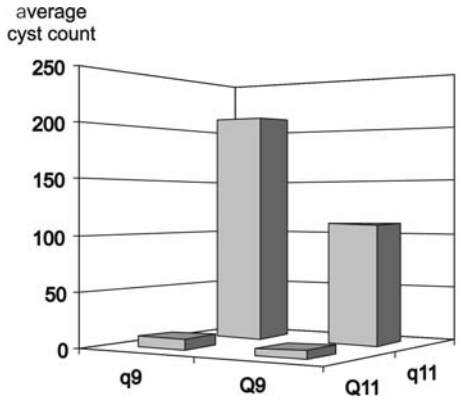
Figure 3: Histograms depicting the distributions of the average natural logarithms of cyst counts (+1) of the individuals of mapping population RHAM-061 after inoculation with *G. pallida* population Rookmaker (Pa3). A: Histogram of all the individuals of mapping population RHAM-061. Black = genotypes tentatively assigned as resistant; on average less than 8 cysts. Grey = genotypes not assigned; on average between 9 and 24 cysts. White = genotypes tentatively assigned as susceptible; on average more than 25 cysts. This classification of genotypes into the classes resistant/susceptible allowed mapping of the locus between flanking AFLP markers. B: Histogram depicting the distribution of RHAM-061 genotypes where both flanking AFLP markers eACTmCAA_174 and eAGAmCTG_222 suggest the presence of resistance. Clones with recombination events between the markers were excluded from the figure. C: Histogram depicting the distribution of RHAM-061 genotypes where both flanking AFLP markers eACTmCAA_174 and eAGAmCTG_222 suggest the absence of resistance. Clones with recombination events between the markers were excluded from the figure.

In order to validate the classification and the resulting map location, the flanking AFLP markers can be used to predict in retrospect the absence or presence of the resistance gene. When classifying on the basis of the flanking AFLP markers eACTmCAA_174 and eAGAmCTG_222 linked with resistance, the resistant group had on average 9.0 cysts, while the susceptible group had on average 148.9 cysts. The expected resistance, based on the prediction, was compared with the observed phenotypes (Figure 3B, C). Judging on the basis of the flanking AFLP markers, eight genotypes out of a total of 140 genotypes showed cyst counts that were incongruent with the expected presence/absence of the resistance gene, and were putatively misclassified. Four of these misclassifications concerned genotypes with no recombination event between the markers on the long arm of maternal chromosome *11*. The resistance phenotype of these descendants should be considered as false positives or negatives, without consequences for the position of the *R*-gene. The other four descendants can be best explained by assuming misclassification of the absence/presence of the flanking AFLP marker eAGAmCTG_222.

Interaction between *GpaXI*$^{l}_{tar}$ and the QTL on chromosome *9*

After identifying the two resistance QTL on chromosome *9* and *11*, the magnitude of the individual effects and the possible interaction between the two QTL was analysed using ANOVA. The flanking AFLP markers eACTmCAA_174 and eAGAmCTG_222 linked with the *GpaXI*$^{l}_{tar}$ locus were used as explaining variable, indicative for the *R*-gene on chromosome *11*. AFLP marker eACAmCCA_26 showed

the closest linkage with the QTL on chromosome *9* and was used as explaining variable indicative for the minor-effect QTL. Analysis of variance demonstrated (as expected) significant main effects for both QTL, but also a significant interaction effect between the QTL of chromosome *9* and *11*. This interaction effect is best explored by illustrating the 2 x 2 interaction of both QTL alleles in Figure 4, where the cyst counts of these four groups are shown. The group with the susceptible allele for both $GpaXI^{l}_{tar}$ and the chromosome *9* QTL showed an average cyst count of 210 cysts (n=29). The group with the resistant allele of this QTL but the susceptible allele of $GpaXI^{l}_{tar}$ had an average cyst count of 109 (n=32). This represents a significant decrease of 48% in the development of cyst numbers (2-sided t-test on ln (cyst count+1), df = 43, p=0.00012). In the presence of $GpaXI^{l}_{tar}$ the minor QTL had no statistically significant effect (2-sided *t*-test on ln (cyst count+1), df = 75, p=0.14). If the combination of the two QTL would have result in an additive effect, the expected average cyst count should be 5. Therefore it is concluded that $GpaXI^{l}_{tar}$ displays a dominant epistatic interaction over the minor QTL.



QTL composition of groups	average cyst count	standard error of the mean
qq qq	210.1	19.1
qq Q9q	109.3	17.0
Q11q qq	9.9	1.9
Q11q Q9q	6.7	0.9

Figure 4: The reproduction of *G. pallida* Rookmaker (Pa3) in relation to the genetic composition of potato genotypes of the RHAM-061 mapping population. The heights of the bars represent the average number of *G. pallida* Rookmaker (Pa3) cysts in the four QTL offspring classes. The presence or absence of $GpaXI^{l}_{tar}$ is determined by flanking AFLP markers eACTmCAA_174 and eAGAmCTG_222. Presence/absence of the QTL on chromosome *9* was identified by AFLP marker eACAmCCA_26. Q9 = resistant allele of the QTL on chromosome *9*. q9= susceptible allele of the QTL on chromosome *9*. Q11= resistant allele of $GpaXI^{l}_{tar}$. q11= susceptible allele of $GpaXI^{l}_{tar}$.

Anchoring of *GpaXI*$^{l}_{tar}$ to a well known *R*-gene cluster

The long arm of potato chromosome *11* is known to harbour several resistance genes. In order to determine the position of *GpaXI*$^{l}_{tar}$ relative to these genes, an attempt was made to generate a better resolution and to add reference markers, which are known to reside close to the *R*-gene cluster.

To screen for recombinants, DNA of 324 newly sown genotypes from RHAM-061 was genotyped with the flanking AFLP markers eACTmCAA_174 and eAGAmCTG_222. Recombinant genotypes were identified and subjected to a disease test. For 32 genotypes we obtained phenotypic data allowing identification of the position of *GpaXI*$^{l}_{tar}$ relative to markers GP163 and FEN427. In the complete population of 324, no recombination events were detected between markers GP163 and FEN427. Both markers mapped 1.3 cM proximal of *GpaXI*$^{l}_{tar}$. The position of GP163 and FEN427 relative to *GpaXI*$^{l}_{tar,}$ confirms that *GpaXI*$^{l}_{tar}$ maps to a well known *R*-gene cluster on the long arm of chromosome *11*. The resulting genetic map is presented in Figure 5, also showing reference maps from literature used for anchoring purposes. Seventeen of the 324 offspring clones (= 5 %) were excluded from map construction. In these plants we observed two recombination events at close distance, suggesting data point(s) that are in conflict with both their flanking markers (singletons). In view of chiasma interference these events are more likely to represent data error than true recombination events and can be removed safely without influencing marker order (van Os *et al.* 2005b). Even if these were real recombination events, the map distances would be hardly affected in view of the total population size of 324 offspring.

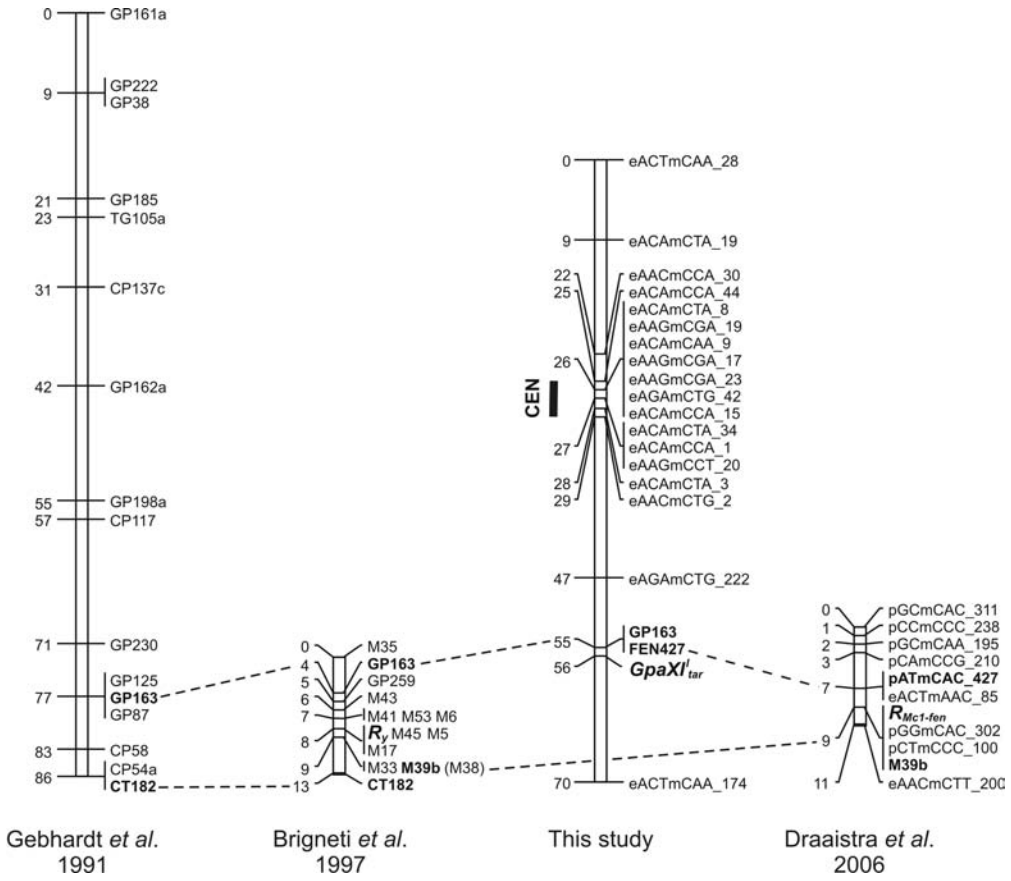


Figure 5: The genetic map of maternal chromosome *11* of mapping population RHAM-061 showing the *GpaXI*$^{I}_{tar}$ locus in connection to chromosome *11* reference maps from literature used for anchoring purposes. Chromosome orientation is according to Dong *et al*. (2000).

Discussion

The inheritance of resistance to the white potato cyst nematode *G. pallida* has initially been regarded as complex (Dale and Phillips 1982; Ross 1986). With the aid of molecular markers it has been demonstrated that the allegedly complex resistance to *G. pallida* is simpler, because in many cases large-effect QTL have been identified.

For example, the QTL *Grp1, Gpa, Gpa5* and *GpaV*$^{s}_{spl}$ (Caromel *et al.* 2005; Kreike *et al.* 1994; Rouppe van der Voort *et al.* 1998a; Rouppe van der Voort *et al.* 2000) invariably accounted for more than 45% of the phenotypic variance. In this study two loci have been mapped: a large-effect QTL derived from the wild *Solanum* species *S. tarijense* BGRC 24717, as well as a minor-effect QTL on potato chromosome *9*.

Identification of the large-effect resistance locus *GpaXI*$^{l}_{tar}$

A large-effect QTL has been identified on potato chromosome *11* explaining 81.3 % of the phenotypic variance of the disease test. In view of the magnitude of the explained variance, the QTL was Mendelised. This allowed to estimate the genetic distances between the locus and the flanking molecular markers and to place the locus name on the genetic map. Usually, a broad QTL interval does not offer precise ordering information of markers relative to the QTL, but in our case the order of the *R*-gene and the markers is regarded as a stable order, well supported by recombination events.

In the studied mapping population RHAM-016, the clones with the resistance allele *GpaXI*$^{l}_{tar}$ (as predicted by the flanking markers) developed on average 9.9 cysts, whereas clones without any resistance allele developed on average 210 cysts. This constitutes a relative decrease of 95 % in cyst count. The level of resistance obtained with *GpaXI*$^{l}_{tar}$ is therefore partial and not absolute. This non-absolute level of resistance seems to be common for most of the identified major loci involved in *G. pallida*. As discussed by Rouppe van der Voort *et al.* (1998a; 2000), the number of newly formed cysts in spite of the presence of a major *R*-gene, is likely due to heterogeneity in the *G. pallida* population Rookmaker (Pa3) at the respective (a)virulence gene. A single *R*-gene which operates on a gene-for-gene basis will confer partial resistance against the population as a whole, whereas on the level of the individual, the *R*-gene will confer absolute resistance against the matching avirulent genotype. Alternatively, it is also possible that the resistance mechanism of the *R*-gene in itself is not absolute. This has been demonstrated for the *H1* gene, where always a small number of cysts are formed on *H1* resistant plants after inoculation with a homozygous avirulent line of *G. rostochiensis* (Janssen *et al.* 1990). These cysts that are formed by a non-absolute resistance mechanism of a major *R*-gene are known as “escapers” and do not imply any kind of evolution of the nematode population towards increasing virulence. Whether the newly formed cysts on

GpaXI$^{l}_{tar}$ resistant plants have been caused by the heterogeneity of the test population or by the strength of the *R*-gene itself can only be determined by testing the *R*-gene with a homozygous avirulent nematode population.

The resistant *S. tarijense* hybrid, which was used as parent of the mapping population showed an average cyst count of 2. Even when both resistance factors were present, the major locus *GpaXI*$^{l}_{tar}$ and the minor QTL on chromosome *9*, offspring clones did not achieve the same resistant level as their resistant parent. Apparently, there is an interaction between the *R*-gene(s) and the genetic background in which an *R*-gene is introgressed.

Identification of a small-effect resistance locus on chromosome *9*

Besides the major QTL on chromosome *11*, a minor QTL was detected on chromosome *9*. At this moment we can only speculate on the function of this QTL.
The minor QTL could represent a classic NBS-LLR gene, but also a factor in non-necrogenic resistance, for instance in the formation of hatching substances. Lastly, the QTL could represent a locus involved in the size of the root system. As demonstrated by Kreike *et al.*(1994) a root size QTL will initially appear as a QTL for resistance. The dominant epistatic interaction of *GpaXI*$^{l}_{tar}$ over the minor QTL does not exclude one of the three possible explanations.

Both loci map to well known *R*-gene clusters

Two CAPS markers GP163 and FEN427 co-localised and mapped 1.3 cM proximal of *GpaXI*$^{l}_{tar}$. Marker GP163 was previously positioned at a distance of 3.7 cM proximal to potato virus Y resistance gene Ry_{sto} on chromosome *11* (Brigneti *et al.* 1997). AFLP marker pATmCAC_427, on which CAPS marker FEN427 was based, was previously used to map nematode resistance $R_{Mc1\text{-}fen}$ on chromosome *11* and mapped 2 cM proximal of $R_{Mc1\text{-}fen}$ (Draaistra 2006). Therefore we postulate that *GpaXI*$^{l}_{tar}$ is located in one and the same *R*-gene cluster, which is already comprising three nematode resistance genes $R_{Mc1\text{-}blb}$, $R_{Mc1\text{-}fen}$, $R_{Mc1\text{-}hou}$, (Draaistra 2006), as well as several more resistance genes against viruses and fungi (Gebhardt and Valkonen 2001). From this *R*-gene cluster only *N* (Whitham *et al.* 1994) has been cloned, suggesting that *GpaXI*$^{l}_{tar}$ could represent a TIR-NBS-LRR gene with homology to *N*. Future research may demonstrate the relation between *GpaXI*$^{l}_{tar}$ and the other nematode resistance genes in this cluster. Each of these nematode resistance genes

could be involved in recognition of distinct effector molecules, but might also represent a casus similar to the *Mi* gene, recognising the nematode *Meloidogyne incognita* and the aphid *Macrosiphum euphorbiae* (Rossi *et al.* 1998; Vos *et al.* 1998).

Likewise, the minor QTL described in this study maps in close proximity to a known *R*-gene cluster. This cluster on chromosome *9* comprises the *Sw-5* resistance gene (Brommonschenkel *et al.* 2000), a homologue of *Mi*. Interestingly, this cluster is already implicated in *G. pallida* resistance, as the small-effect *Gpa6* QTL was mapped here (Rouppe van der Voort *et al.* 2000). The coincidence of finding the minor QTL at a position indistinguishable from the *Gpa6* locus prompted us to verify the pedigree of our mapping population for the putative introgression segments of *S. vernei*. In Figure 1, a crossing parent MON-46 is shown, a dihaploid of cv. Mondial. This parent is susceptible for *G. pallida*, but it has *S. vernei* in its pedigree. Therefore it remains inconclusive if the minor QTL originates from *S. tarijense* or represents a gene identical by descent to *Gpa6* from *S. vernei*. Thus, we have refrained from giving the minor-effect QTL discovered on chromosome *9* a new name.

Mapping strategy

The identification of the genetic locus involved in a phenotypic trait can be achieved with various mapping strategies, but a minimal requirement is the ability to align the newly mapped locus with previous linkage studies. This can be achieved with single copy locus specific markers, but in this study the high multiplex efficiency of AFLP fingerprinting was used. With 14 *Eco*RI/*Mse*I AFLP primer combinations, 518 marker loci were obtained, which was sufficient to cover all twelve maternal and paternal linkage groups, as well as markers bridging the homologous parental linkage groups. Our strategy specifically exploited the principle that AFLP fragments of the same mobility on gel represent the same genetic map position, because of DNA sequence homology of the underlying DNA fragment captured in the AFLP fingerprint (Rouppe van der Voort *et al.* 1997b). This approach has been successful not only in our own hands with proper control samples in the same AFLP gel image (Rouppe van der Voort *et al.* 1997a), but also across labs (Bradshaw *et al.* 2006). The approach followed in this study, not only used the well known reference genotype RH89-039-16 as a reference sample in AFLP fingerprinting, but it also served as susceptible crossing partner. The resistance locus however segregated from a wild

species donor. The validity of locus-specificity of AFLP markers rapidly disappears with increasing taxonomic distance; as increasing taxonomic distance results in increasing AFLP fingerprint dissimilarity, where only insignificant coincidental co-migration remains. The obvious success to align the introgression of *S. tarijense* with the potato reference maps is due to the BC1 structure of the mapping population. The *S. tuberosum* derived AFLP alleles in the resistant parent are linked in repulsion phase with the *S. tarijense* derived AFLP and resistance alleles, and allowed the precise mapping of the locus irrespective of genetic dissimilarity between the wild species and potato.

Nomenclature of nematode resistance genes

At this moment the nomenclature of genes involved in nematode resistance is without consensus (Gebhardt and Valkonen 2001). On the one hand there is a series of major and minor-effect QTL against *G. pallida* with names ranging from *Gpa*, *Gpa2* until *Gpa6*, which are numbered successively following their order of identification. Likewise a series of *R*-genes and QTL against *G. rostochiensis* are known: *H1*, *Gro1*, *Gro1.2, Gro1.3, Gro1.4* and *GroV1* (Gebhardt and Valkonen 2001). Although such names nicely reflect history of science, it is hardly informative on the wild species origin, the resistance spectrum to various pathotypes, or the genetic location of the gene.

At this moment we wish to follow the syntax of Caromel *et al.* (2005) [pathogen species, potato linkage group (roman), long/short arm (superscript), source species (subscript)], and propose to name this *G. pallida* resistance $GpaXI^{l}_{tar}$. At the 'Symposium on the Molecular Biology of the Potato' held in 1998 at Bogensee, Germany, two large-effect QTL have been mentioned. An abstract by Wolters *et al.* (1998), describes *Gpa3* on chromosome *11* derived from *S. tarijense* and *Gpa4* on potato chromosome *5* derived from *S. sparsipilum*. We propose that *Gpa3* and *Gpa4* will no longer be used in scientific literature. *Gpa3* was mapped in an F1 segregating population different from our population. But since the same BGRC accession has been used as a source, it is very plausible that $GpaXI^{l}_{tar}$ is identical to *Gpa3*. Whereas *Gpa3* and *Gpa4* have not yet entered the peer-reviewed literature, *Gpa4* has already caused some confusion as it appeared in the review by Gebhardt and Valkonen (2001) where *Gpa4* was used for the unnamed QTL on potato chromosome *4* segregating from SCRI clone 12601ab1 (Bradshaw *et al.* 1998). The most plausible name for this

QTL is the *H3* gene from *Solanum tuberosum* spp. *andigena* CPC2802 (p.115 http://www.scri.ac.uk/scri/file/fullannualreports/annual_report_2001.pdf).

Practical value of the *G. pallida* (Pa3) resistance gene *GpaXI*$^{l}_{tar}$ in potato breeding

The resistance level of *GpaXI*$^{l}_{tar}$ (a relative decrease of 95 % in cyst count) is comparable with the level conferred by *Grp1* (Rouppe van der Voort *et al.* 1998a) and *Gpa5* (Rouppe van der Voort *et al.* 2000); both resistance genes that are presently used in commercially grown PCN resistant cultivars. We therefore believe that *GpaXI*$^{l}_{tar}$ will be an equally valuable gene for the development of PCN resistant cultivars.

In contrast to the limited durability of *R*-genes in many other plant-pathogen interactions, several aspects of the potato – PCN interaction raise the support of a relatively longer durability of the major resistance genes. The multiplication rate and the spread of PCN are limited and the time between generations can be up to 4 years for normal crop rotation. Changes towards new virulent PCN types are therefore likely to be slow. Furthermore the positive selection of virulent factors will be countered by the obligate sexual reproduction with genetically heterogeneous males.

Nowadays numerous PCN resistant cultivars are available. However because of the partial effect of the used *R*-genes a wide crop rotation is still required. As discussed before, the partial effect of the resistance genes can be explained by the genetic heterogeneity of the *G. pallida* field populations and by "escapers" that are caused by an ineffective resistance mechanism. The ideal situation for potato growers would be an absolute resistance in one cultivar. Possibly such an absolute resistance level could be achieved by combining or "pyramiding" of different *G. pallida* resistance genes. The development of breeding material with more than one allele at a given locus seems ineffective (Brodie and Plaisted 1992). Clones with a combination of major-effect QTL *GpaV*$^{s}_{spl}$ and minor-effect QTL *GpaXI*$^{s}_{spl}$ showed additively lower cyst counts than with the individual QTL (Caromel *et al.* 2005). The same was shown with a combination of major-effect QTL *Gpa5* with minor-effect QTL *Gpa6* (Rouppe van der Voort *et al.* 2000). Both studies showed lower cysts counts when combining a major and a minor QTL, but no absolute resistance. It would be interesting whether

combining major QTL could result in absolute resistance. It seems that a combination of *R*-genes with different resistance spectra should at least make it possible to efficiently tackle the problem of heterogeneity of the *G. pallida* populations.

Acknowledgements

We kindly acknowledge Petra Wolters, Jeroen Rouppe van der Voort and Jan Draaistra for earlier contributions to the research. The photograph shown in Figure 2 was kindly provided by Nico Rookmaker.

Chapter 5

Pyramiding of *Meloidogyne hapla* resistance genes in potato

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Abstract

High levels of resistance against *Meloidogyne hapla* have been identified in wild species of tuber-bearing potatoes, but only QTL with partial effect have been identified so far in back crosses with cultivated potato. This study was designed to test if pyramiding of two previously identified resistance genes, $R_{Mh\text{-}tar}$ and $R_{Mh\text{-}chc}A$, will result in improved , or even an absolute level of resistance. $R_{Mh\text{-}tar}$ and $R_{Mh\text{-}chc}A$, introgressed from the wild tuber bearing potato species *Solanum tarijense* and *S. chacoense* were combined in a segregating diploid *S. tuberosum* population. With the aid of AFLP markers, descendants from this segregating population were classified into four groups, carrying no *R*-gene, with only $R_{Mh\text{-}tar}$, with only $R_{Mh\text{-}chc}A$, and a group with the pyramided $R_{Mh\text{-}tar}$ and $R_{Mh\text{-}chc}A$. Upon inoculation with *M. hapla* isolate Bovensmilde, the group containing only $R_{Mh\text{-}chc}A$ showed a decline of 88 % in average developed egg masses compared to the group without $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}tar}$. The group of genotypes containing only $R_{Mh\text{-}tar}$, but not $R_{Mh\text{-}chc}A$, showed a decline of 55% in developed egg masses compared to the group without $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}tar}$. Unfortunately, the latter effect of $R_{Mh\text{-}tar}$ was not significant. The effect of both loci, $R_{Mh\text{-}tar}$ and $R_{Mh\text{-}chc}A$ combined, did not further reduce the number of egg masses compared to the level of $R_{Mh\text{-}chc}A$ alone.

Introduction

Compared to other potato diseases such as late blight or potato cyst nematodes, infection with the root knot nematode species *Meloidogyne hapla* is less noticeable. The damage caused by this pathogen in potato can be expressed only in yield reduction. However, in extremely infected soils the yield reduction can amount up to 70% (Macguidwin and Rouse 1990). Crop rotation, as a strategy to reduce disease pressure, is less practicable to control *M. hapla* infection because of its broad host spectrum. *M. hapla* propagates on many dicotylenous plants and will therefore increasingly infect following crops.

While a number of resistance genes have been incorporated in cultivars, primarily against late blight, viruses, wart disease and potato cyst nematodes, no *M. hapla* resistant cultivars are currently available. The deployment of cultivars resistant against cyst nematodes allowed suspending soil fumigation with nematicides. Therefore a shift in the nematode populations towards *M. hapla* should be expected in the immediate future.

Introgression of resistance genes from wild *Solanum* species is viewed as an efficient solution to reduce *M. hapla* infection. A broad range of wild tuber-bearing *Solanum* species has been tested for *M. hapla* resistance by Janssen *et al*. (1996b). High levels of resistance have been identified in 14 different *Solanum* species. In these studies it already became apparent that the resistance to *M. hapla* in these wild species would not lead to absolute immunity in potato. Draaistra *et al.* (2006) studied the inheritance of *M. hapla* resistance originating from *S. chacoense* (accession BGRC 18618) and *S. tarijense* (accession BGRC 24717) and localised genetic loci involved in resistance on linkage maps. The major *S. chacoense* QTL, named $R_{Mh\text{-}chc}A$ explained 38% of the phenotypic variance, and mapped to a distal region of a linkage group that has not been assigned to a potato chromosome. The locus involved in resistance originating from *S. tarijense* ($R_{Mh\text{-}tar}$) explained 20% of the phenotypic variance and could be mapped as a qualitative trait locus to a distal location on potato chromosome *7*. Offspring from neither *S. chacoense* nor *S. tarijense* displayed absolute levels of resistance, which is essential to avoid propagation of the pathogen population. At this moment it is not fully understood why the transmission of resistance from highly resistant wild species, only results in back cross offspring with partial resistance to *M. hapla*. This study was designed to test if pyramiding of these two resistance genes $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}tar}$ will result in improved , or even an absolute level of resistance.

Materials and methods

Plant material

The diploid full-sib population RH04-456, pyramiding two loci involved in *M. hapla* resistance, was generated from a cross between clones RH90-011-4 and 87-206-6. Both parents were previously used as parents for the respective mapping populations that allowed the identification and genetic mapping of $R_{Mh\text{-}tar}$ and $R_{Mh\text{-}chc}A$ (Draaistra

2006). The $R_{Mh\text{-}tar}$ donor RH90-011-4 was obtained from an interspecific cross between *S. tarijense* (BGRC 24717 / CGN 18107) and MON-46, a dihaploid clone of the susceptible cultivar Mondial (see Figure 1). The *S. chacoense* resistance from 87-206-6 was first described by Janssen *et al.* (1996a) and later by Draaistra *et al.* (2006). Plant material was propagated to obtain sufficient seed tubers for the disease test.

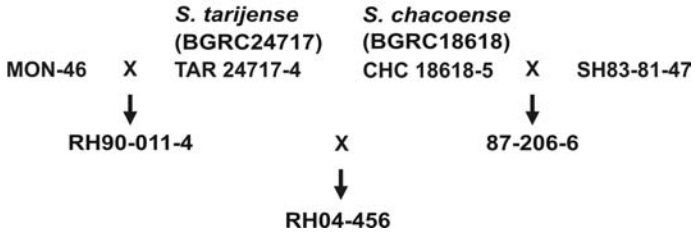

Figure 1: Pedigree of the pyramiding population RH04-456 combining the two loci involved in *M. hapla* resistance $R_{Mh\text{-}tar}$ from *S. tarijense* and $R_{Mh\text{-}chc}A$ from *S. chacoense*.

***Meloidogyne hapla* resistance assay**

112 offspring genotypes of pyramiding population RH04-456 were tested for *M. hapla* resistance. Tubers were planted in 500 ml clay pots containing silversand and a slow release NPK fertiliser (Osmocote). The genotypes were tested for their level of resistance in three replications in a randomised complete block design. Nicola and the resistant parents RH90-011-4 and 87-206-6 were included as control plants. Three weeks later, when the root systems were fully developed and reached through the bottom hole of the pots, the plants were inoculated with *M. hapla* isolate "Bovensmilde" at a concentration of 750 second stage juveniles (J2) per pot. The inoculum "Bovensmilde", is commercially available from RZ Research, Metslawier, The Netherlands. The first two weeks after inoculation, the plants were watered with great care to avoid flushing the juveniles from the pots. Eight weeks after inoculation, the root systems were released from the sand by rinsing with tap water. Egg masses were stained with Phloxine-B (Dickson and Struble 1965) and counted. Variation in the size of the root system was recorded on a scale of 1-5 where 1 = small root system and 5 = large root system.

DNA extraction

Genomic DNA from the parents and the offspring population RH04-456 was isolated from young leaf material essentially as described by Stewart and Via (1993). Leaf material was ground with steel balls using a Retsch machine (Retsch Inc., Haan, Germany) in the CTAB-buffer, with volumes adapted to 2 ml deep 96-wells Costar plates (Corning Inc., Corning, NY, U.S.A.). DNA was visualised on a 1 % agarose gel to check its integrity. DNA concentrations were measured with a NanoDrop™ ND 1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA).

Genotyping with AFLP™ markers

The genetic maps constructed in the mapping studies of $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}tar}$ were solely constructed with AFLP markers (Draaistra 2006). Therefore, the same AFLP markers linked to $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}tar}$ were used to analyse the genetic composition of the offspring genotypes of the pyramiding population. AFLP was performed according to Vos *et al.* (1995). Fluorescently labelled AFLP fragments were visualized on a denaturing polyacrylamide gel using a NEN® Global Edition IR2 DNA Analyzer (LI-COR® Biosciences, Lincoln, NE). The AFLP fragments were scored visually for absence/presence. Three primer combinations per resistance gene were tested. To assess the presence or absence of $R_{Mh\text{-}chc}A$ the primer combinations eACAmCCT, eACTmCAT, and eAGAmCTC were analysed, and for $R_{Mh\text{-}tar}$: pAGmAAG, pAGmAGG (2 marker loci), and pAGmAGA were used.

Of all 7 markers tested, only three markers generated an informative polymorphic pattern diagnostic for the presence of the resistance locus: eACAmCCT_383 and eACTmCAT_165 for $R_{Mh\text{-}chc}A$ and pAGmAGA_221 for $R_{Mh\text{-}tar}$ (see Figure 2). Marker pAGmAGA_221 was previously known as pAGmAGA_226 (Draaistra 2006). The fragment mobility of 226 was obtained with a ^{33}P labelled *Eco*RI primer followed by autoradiography, whereas the mobility of 221 refers to the same fragment obtained with fluorescently labelled primers and separation on a LICOR system. The congruence of fragment mobilities between these two AFLP methods cannot be predicted in advance. One marker (pAGmAAG_111) could not be traced back, possibly due to methylation of the *Pst*I site or a shift in mobility, where the fragment is hidden underneath other AFLP fragments.

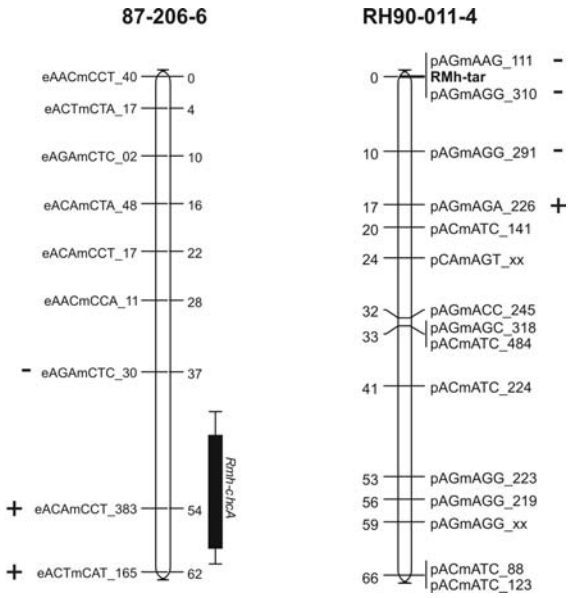

Figure 2: Linkage groups indicating the position of AFLP markers and the position of $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}tar}$ (Draaistra 2006). The diagnostic value of the marker pattern in this pyramiding study is indicated with a plus or minus sign, where the sign indicates diagnostic or uninformative segregation pattern respectively.

Statistical analysis

Statistical analyses were performed with MS-Excel and Genstat 10th Edition. The number of egg masses counted per root system were transformed by taking the natural logarithm of the (egg mass number +1), to obtain a more uniform distribution of the variance. The heritability was estimated as follows: $h^2 = \sigma^2_g / (\sigma^2_g + \sigma^2_e/n)$, where n is the number of replications, σ^2_e is the expected mean square of the residuals and σ^2_g is retrieved from the one-way ANOVA table as follows: $E(MS)genotypes = n\sigma^2_g + \sigma^2_e$. The individual and combined effect of $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}tar}$ was analysed with a General ANOVA. The size of the root system was taken as a covariate in ANOVA and a block structure was included in the model of: (repetition*genotype)/plant.

Results

Genotyping of pyramiding population RH04-456

The 148 offspring genotypes of the pyramiding population RH04-456 were genotyped with markers eACAmCCT_383 and eACTmCAT_165 to infer the presence of $R_{Mh\text{-}chcA}$, while pAGmAGA_221 was used for $R_{Mh\text{-}tar}$. Due to missing values as well as recombination events between markers eACAmCCT_383 and eACTmCAT_165, 10 and 26 genotypes respectively were excluded from further analysis. In total, 112 genotypes remained for testing in the disease experiment. The pyramiding population could be differentiated into 4 groups: 30 without *R*-genes, 27 with $R_{Mh\text{-}tar}$ present, 26 with $R_{Mh\text{-}chcA}$ present, and 29 containing both *R*-genes $R_{Mh\text{-}tar}$ + $R_{Mh\text{-}chcA}$ pyramided. The observed numbers of genotypes in each of the four groups complied with the expected 1:1:1:1 segregation ratio (χ^2 =0.357; p=0.948).

***M. hapla* disease experiment**

To test for resistance to *M. hapla*, 112 genotypes were inoculated with isolate "Bovensmilde". The number of egg masses developed in the root system of individual plants of the pyramiding population differed between 0 and 221. An example of egg masses stained red with Phloxine-B on a susceptible plant is shown in Figure 3. The parental clones used as control displayed a high level of resistance. The *S. chacoense* × *S. tuberosum* hybrid 87-206-6 developed on average 1 egg mass per root system, the *S. tuberosum* × *S. tarijense* hybrid RH90-011-4 on average 3 egg masses. The susceptible control cv. Nicola developed on average 127 egg masses. The numbers of egg masses were converted by a natural logarithm to reduce the variance before further data analysis. According to ANOVA, the genotypes showed significant differences in their number of egg masses (F-ratio = 8.12; df = 530; $p < 0.001$). The heritability of the offspring population was 0.92.

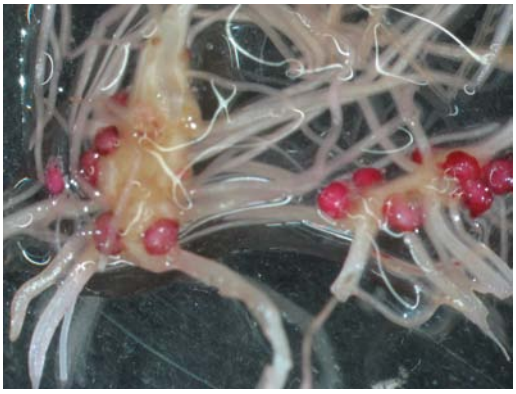

Figure 3: *M. hapla* egg masses on the root system of a susceptible potato plant.

The individual and joint effect of the loci $R_{Mh\text{-}tar}$ + $R_{Mh\text{-}chc}A$ on the level of resistance against *M. hapla*

The size of the root system significantly influenced the number of egg masses (*F*-ratio = 6.06; df = 1; $p < 0.015$), and was taken as a covariate in further analysis.

The pyramiding population, differentiated into four groups based on flanking molecular markers, allowed estimating the effect of the loci $R_{Mh\text{-}tar}$ + $R_{Mh\text{-}chc}A$. The group without $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}tar}$ developed on average 25.8 egg masses (see Table 1). Figure 4 shows the distribution of mean egg masses per genotype for each group. The group of genotypes containing $R_{Mh\text{-}tar}$, but not $R_{Mh\text{-}chc}A$, developed on average 11.8 egg masses, which means a decline of 55% in developed egg masses compared to the group without $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}tar}$. However, according to ANOVA, this effect of $R_{Mh\text{-}tar}$ on the number of egg masses is not significant (F ratio = 1.02; df = 1; p = 0.315).

The group containing $R_{Mh\text{-}chc}A$, but not $R_{Mh\text{-}tar}$, developed on average 3.1 egg masses. The effect of $R_{Mh\text{-}chc}A$ is highly significant (F ratio = 23.29; df = 1; $p < 0.001$) and offers a reduction of 88 % of the number of egg masses as compared to the group without $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}tar}$.

Table 1: Analysis of the individual and joint effect of the loci $R_{Mh\text{-}tar}$ + $R_{Mh\text{-}chc}A$ on the level of resistance against *M. hapla*. 1)The number of genotypes per marker class (n) 2) the average number of egg masses per root system, 3) standard error of the mean, average ln(egg mass +1) is shown for the four groups of pyramiding population RH04-456 containing no *R*-gene, one *R*-gene or both *R*-genes $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}tar}$. * The suffix a or b denote a statistically significant difference between groups.

group	**n**[1]	**Average number of egg masses**	**Standard error of the mean**	**Average ln (egg mass + 1)***
No *R*-gene	30	25.8	4.17	2.136 [a]
$\boldsymbol{R_{Mh\text{-}tar}}$	27	11.8	2.57	1.424 [a]
$\boldsymbol{R_{Mh\text{-}chc}A}$	26	3.1	0.69	0.704 [b]
$\boldsymbol{R_{Mh\text{-}chc}A + R_{Mh\text{-}tar}}$	29	3.0	0.57	0.759 [b]

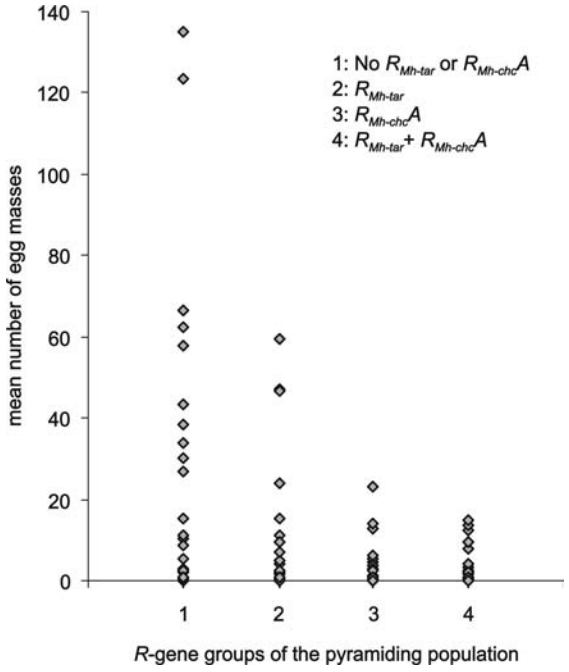

Figure 4: Distribution of the mean number of egg masses per genotype over the four groups as determined by molecular markers flanking the $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}tar}$ loci.

When both *R*-genes are present, the average number of egg masses is 3.0 (ln (egg mass +1) = 0.76). Compared to the effect of 3.1 egg masses caused by $R_{Mh\text{-}chc}A$ alone, the joint effect of both loci, $R_{Mh\text{-}tar}$ and $R_{Mh\text{-}chc}A$ does not further reduce the number of egg masses. This is also evident from the ANOVA, where an interaction between $R_{Mh\text{-}tar}$ and $R_{Mh\text{-}chc}A$ (F ratio = 3.06; df = 1; p = 0.083) was observed. Hence the locus $R_{Mh\text{-}chc}A$ shows an epistatic interaction over the $R_{Mh\text{-}tar}$ locus.

Discussion

The objective of this research was to investigate if pyramiding of two *M. hapla* resistance genes allowed a further reduction of the number of egg masses as compared to the reduction obtained by the separate QTL for resistance. Due to the large variation in egg masses of the groups containing $R_{Mh\text{-}tar}$, and the relatively small group sizes, the effect of $R_{Mh\text{-}tar}$ was not significant. The average decline of 55% in egg masses suggests however that the effect of $R_{Mh\text{-}tar}$ is probably real. Due to the lack of statistical significance of the contribution of $R_{Mh\text{-}tar}$, we can only now speculate on the effect of pyramiding $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}tar}$. The results of this experiment seem to suggest that pyramiding $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}tar}$ does not lead to an additional reduction in the number of egg masses, and hence the level of resistance has not improved.

This study also provides a validation of the value of the $R_{Mh\text{-}chc}A$ locus. The $R_{Mh\text{-}chc}A$ locus was first identified as a QTL explaining 38% of the phenotypic variance and causing a 70% reduction of the number of egg masses, relative to susceptible offspring, in a BC1 mapping population (Draaistra 2006). In this study the effect of the $R_{Mh\text{-}chc}A$ locus was even stronger causing 88% reduction. Therefore, the value of this gene for potato breeding is beyond doubt, although cultivar registration authorities may require a higher level of reduction before a cultivar can be called resistant according to their criteria.

The $R_{Mh\text{-}tar}$ locus was initially identified in a BC1 mapping population, where a clear-cut Mendelian segregation of resistance was observed. Nevertheless, the $R_{Mh\text{-}tar}$ locus explained only 20% of the phenotypic variance in the mapping population, which demonstrates the great variability in the number of egg masses that can develop on an

individual plant. This great variability also had a great impact on the statistical analysis of the QTL effects in this experiment. Although a clear 50% reduction of egg masses was observed, the effect was not significant. Hence, the validation of $R_{Mh\text{-}tar}$ was not achieved, but $R_{Mh\text{-}tar}$ probably remains a valuable gene for breeders. In retrospect, to obtain statistical significance for small-effect QTL the offspring size of the experimental population should have been larger than the current 112 descendants, distributed over 4 classes with 26 – 30 plants per class.

In addition to the trait variability, the genetic distance of 17 cM between $R_{Mh\text{-}tar}$ and the diagnostic AFLP marker pAGmAGA_221 posed a second complication. Because of this genetic distance it is likely that some genotypes have recombination events between AFLP marker pAGmAGA_221 and resistance locus $R_{Mh\text{-}tar}$, and thus not containing the resistance locus, incorrectly entered the $R_{Mh\text{-}tar}$ group. The decline of 55% caused by $R_{Mh\text{-}tar}$ is therefore likely to be an underestimation of the actual effect. The same reasoning holds true of course for the groups without $R_{Mh\text{-}tar}$. In retrospect we should have used the AFLP markers that mapped closer to $R_{Mh\text{-}tar}$, but of which the diagnostic allele was found in both parents of the pyramiding population. These markers were not tested, but could have resulted in a 3:1 segregating AFLP polymorphism, where marker presence would not be informative. The offspring of the pyramiding population without the diagnostic band would certainly not have $R_{Mh\text{-}tar}$. Thus with a marker that is heterozygous in both parents, a theoretic 25 % of the offspring could still be genotyped. In this study, it could have eliminated 25 % of the wrongly classified genotypes with a recombination event between the used AFLP marker pAGmAGA_221 and resistance locus $R_{Mh\text{-}tar}$.

The heritability of 92% obtained in this study, clearly demonstrates that the experimental error between plants within a genotype is very low. Hence, the resistance level of an individual genotype has been determined with high accuracy. The high variance between genotypes within *R*-gene classes caused the lack of statistical significance of the effect of $R_{Mh\text{-}tar}$. A striking observation is the discrepancy between the high heritability of this experiment with the modest explained variance of $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}tar}$ as observed in the initial mapping studies of Draaistra (2006). Usually the gap between high heritability and low explained QTL variance is ascribed to genetic background. This could point to the putative significance of $R_{Mh\text{-}chc}B$, also known to segregate in this population. More likely

however, this is another confirmation that recombination between the marker and $R_{Mh\text{-}tar}$ has compromised the statistical significance of this experiment.

In retrospect, the experimental design of this study should not have neglected the contribution of the $R_{Mh\text{-}chc}B$ locus which descends from the resistant parent 87-206-6. This small-effect QTL explained only 9.6 % of the phenotypic variance (Draaistra 2006), which was deemed insignificant and negligible. However, it might have had its own unique value in this pyramiding study. This unique value depends on the mutual complementarities of the *R*-gene and the unique composition of each different *M. hapla* population.

The experiment may also have been compromised by the difference between the *M. hapla* nematode populations. Population Hi from the location "Smilde" was used to detect $R_{Mh\text{-}chc}A$, and population Hb from the location "Zwaanshoek" was used to detect $R_{Mh\text{-}tar}$ (Draaistra 2006). These populations have been characterised by Van der Beek *et al*. (1998). In this pyramiding study a different population from "Bovensmilde" was used, which was commercially available from RZ Research, Metslawier, The Netherlands. This issue demonstrates the interdependency of plant geneticists and nematologists and the necessity to maintain public accessible collections of well characterised research materials for a reasonable price.

The nature of the nematode population has great implications for the research of plant geneticist. At this moment there is little information on the composition and allele frequency of avirulence genes within *M. hapla* populations. Differences between populations can greatly affect the identification of loci involved in nematode resistance and the estimates of the size of the QTL-effect. A small-effect QTL such as $R_{Mh\text{-}tar}$ might appear as a large-effect QTL or as a single *R*-gene with absolute effect, depending on the composition of the nematode population. The ease at which nematode populations can change their composition was demonstrated by Janssen *et al*. (1998) who could develop *R*-gene breaking *M. chitwoodi* populations in one generation, by selection of largely non-breaking isolates and subsequent multiplication on tomato. Furthermore, the efforts required to avoid admixture of *M. hapla* populations with individuals from *M. chitwoodi* and *M. fallax* are not trivial.

Genotyping the pyramiding population with the available AFLP markers proved to be problematic. Polymorphic patterns observed in a specific mapping population can not always be expected in other populations. In this pyramiding population the parents of both mapping populations were used to construct the pyramiding population to assure that the marker allele associated with the resistance locus will at least be recognised in the newly generated population. In this study three cases were observed where the diagnostic marker alleles were also observed in the other parent. The diagnostic value of marker alleles across unrelated genotypes or different mapping populations than the mapping population in which the markers were developed can be improved if such an assay infers the haplotypes based on multiple SNPs (Sattarzadeh *et al.* 2006). In the study of Sattarzadeh *et al*. (2006) the allele specific PCR primers were sensitive to SNPs that were diagnostic for a specific haplotype, but even this strategy resulted in a few false positive/ false negative results in spite of the efforts to maximize the diagnostic value of the marker. An alternative PCR strategy is the use of non-allele-specific PCR primers that will amplify all alleles and use DNA polymorphisms in the recognition sites for restriction enzymes. Even if a certain restriction enzyme used for such a CAPS marker analysis (Cleaved Amplified Polymorphic Sequence) does not give polymorphism in the population to be genotyped, it is still possible to find another restriction enzyme that will exploit a DNA polymorphism in another restriction site.

Acknowledgements

We would like to acknowledge Robert Machado for technical assistance in the disease test, Petra van den Berg for laboratory assistance and Chris Maliepaard and Joao Paulo for their statistical advice.

Chapter 6

General Discussion

In this thesis, the experimental work is described in four chapters. Two chapters focus on the identification, mapping and characterisation of a late blight and a nematode resistance gene. The other two chapters focus on the pyramiding of *R*-genes to achieve something more than was offered by the individual *R*-genes. Besides a quantitatively higher level of resistance, the additional value of pyramided *R*-genes could be a prolonged durability and resistance against a broader spectrum of pathogen races. This final chapter aims to discuss the experimental work in a broader context. On the one hand the discussion will connect to fundamental understanding of evolutionary aspects and molecular interactions between elicitor and receptor molecules that determine the long term and short term outcome of plant-pathogen interactions. On the other hand the practical implications for potato breeding and disease resistance management are discussed.

Evolutionary aspects

In potato cultivars already a large number of *R*-genes have been identified, most of them originating from wild potato species. Nevertheless, the need to add more *R*-genes to the potato gene pool remains. Breeding for resistance is a form of man-made crop evolution, causing pathogen evolution. Therefore this discussion is based on several fundamental evolutionary biological themes, namely mutation, selection, migration, and genetic drift. In the case of plant pathogens this translates as follows.
The combination of both past and future mutation events results in a genetically diverse pathogen population. The mutations of the past may reside in the pathogen populations at a low allele frequency, which in a selective environment can result in a quick adaptation of the pathogen. The durability of *R*-genes should be understood in the light of past and future mutation events. At this moment it is unknown if the (lack of) durability of *R*-genes is due to mutations in *Avr*-genes which were already present prior to the introduction of novel *R*-genes. In that case it is obvious to understand the short time in which an *R*-gene has its value. The strong selection pressure imposed on the pathogen population will result in a rapid increase of the allele frequency of the virulence allele. The probability that future mutations will help the pathogen to overcome novel *R*-genes largely depends on the pathogen population size. The number of nematodes in the soil is many factors lower than the number of late blight spores in the air. This may explain why for example the nematode resistance gene *H1*

is still effective, whereas examples of durable late blight *R*-genes are absent. The aspects of migration and drift are also dramatically different for nematodes and late blight. The soil borne nature of nematodes implies that genotypes having certain mutation events remain within highly localised populations, whereas the late blight pathogen populations can travel, mix and mate irrespective of geographical distance.

The aspect of selection of prior and future mutations is best illustrated with the potato - nematode interaction, because of the heterogeneity of nematode populations in the soil. The aspect of selection is not an issue for late blight, because it is close to certainty that new races will emerge, spread and will reach high allele frequencies. In view of the strong evolutionary potential of late blight due to numerically large numbers of individuals, different aspects will be discussed, such as wise strategies to deploy *R*-genes, fitness costs for the pathogen and the probability and putative effects of various mutations.

The characterisation and pyramiding of late blight resistance genes

Molecular indications that weak effect *R*-genes are NBS-LRR

Van der Plank wrote in 1968 that disease resistance could be classified into two types: horizontal and vertical resistance. The vertical resistance was race-specific, absolute and caused by *R*-genes. The horizontal resistance was race-non-specific, partial and not caused by *R*-genes (van der Plank 1968). For a long period of time, these black-and-white definitions prevailed in disease resistance research. However, modern insights must bring more nuances in this paradigm. Partial resistance is often mapped as a QTL in clusters of known *R*-genes (Gebhardt and Valkonen 2001). This suggests that partial resistance has the same molecular basis as *R*-genes. The case of $R_{Pi\text{-}mcd1}$, described in Chapter 2, is one more example of such a partial resistance that maps in an *R*-gene cluster. Furthermore, in studies of rice, a homologue of a major *R*-gene was shown to confer partial levels of resistance. *Xa21* is an *R*-gene that confers race-specific resistance to bacterial blight in rice. The *R*-gene *Xa21D* is a family member of *Xa21*, but has a retrotransposon in the coding region causing the protein to lack a kinase domain. Most likely, *Xa21D* arose by duplication of a progenitor *Xa21* gene

with subsequent integration of the retrotransposon (Song *et al.* 1997). Transformants with *Xa21D* have an identical resistance spectrum as *Xa21*, but have an intermediate level of resistance compared to *Xa21* transformants (Wang *et al.* 1998). This example clearly demonstrates that partial resistance could be caused by mutations in kinase domains of *R*-genes. In line with this theory is the hypothesis proposed by Vleeshouwers *et al*. (2000), explaining the quantitative phenotype of late blight resistance. During late blight attack, it is the amount of time the host cell needs to recognise invading late blight hyphae. Hence, partial resistance is a function of the percentage of cells that can counteract infection in a timely fashion. The reaction time of the *R*-gene product to signal cell death is one of the aspects that could be directly influenced by mutations in the *R*-gene.

Another argument against horizontal non *R*-gene mediated resistance is that until now no significant resistance in potato has ever been described in literature that was for certain not an NBS-LRR type gene, except for the gene for maturity type. It is not clear if the emphasis on NBS-LRR genes for potato – late blight interactions can be generalised to other plant pathogen systems. Research on the barley – *Puccinia hordei* interaction demonstrated that the distribution of *R*-genes did *not* co-localise with QTLs for partial resistance (Qi *et al.* 1998). This suggests an opposite situation to potato – late blight.

Value of weak effect late blight *R*-genes for potato breeding

Although the *R*-genes that only give partial resistance may be less interesting for breeding purposes because they cannot stop a pathogen attack fully, we believe that they might still be still valuable. Weak effect R-genes can delay or slow down the infection. In practical terms this can add an extra week to the growth cycle of potato, thus increasing the yield, before obligatory defoliation. And as will be discussed later on, when pyramiding the weak effect *R*-genes in one cultivar, the level of resistance can be increased and possibly also the durability of the pyramided *R*-genes can be lengthened.

Pyramiding of late blight *R*-genes

During the last century, the appreciation for late blight *R*-genes has fluctuated. After the initial deployment and the breakdown of the major *R*-genes (*R*-genes with large effects) from *S. demissum*, there was a period where *R*-gene free cultivars with

horizontal field resistance were pursued. Appreciation for major *R*-genes against late blight is back again (Allefs *et al.* 2005), as can be inferred from the introduction of new cultivars Toluca and Bionica with late blight *R*-genes from *S. bulbocastanum*, as well as the current efforts to clone *R*-genes. The concern for the lack of durability of late blight *R*-genes has however not diminished; and maybe rightfully so. Pyramiding of *R*-genes can serve multiple goals: to raise the level of resistance, to broaden the spectrum and improve durability. For late blight, the major concern is without a doubt, the lack of durability. Pyramiding late blight *R*-genes can however also benefit the level of resistances in the practical field situation.

In Chapter 3, we have studied the effect of pyramiding two late blight *R*-genes which confer different levels of quantitative resistance. The two resistance genes, $R_{Pi\text{-}mcd1}$ and $R_{Pi\text{-}ber}$, were introgressed from the wild tuber bearing potato species *S. microdontum* and *S. berthaultii* and were combined in a segregating diploid *S. tuberosum* population. Data from a field experiment revealed that genotypes with both *R*-genes $R_{Pi\text{-}mcd1}$ and $R_{Pi\text{-}ber}$, showed an additive higher level of resistance compared to genotypes with only one of the *R*-genes. This result suggests that potato breeding can indeed benefit from pyramiding late blight *R*-genes.

It is always difficult to make predictions, in particular about the future. Besides raising the level of resistance, pyramiding *R*-genes might contribute to the durability of resistance (Nelson 1972). Although the durability of *R*-genes lies beyond the scope of this thesis, it is possible to speculate. There are two processes that determine that an *R*-gene loses its functionality. Firstly, an individual of the pathogen population must mutate in such a way that the avirulence product is no longer recognised by the *R*-gene product. Secondly, by selection the population will shift towards the virulent biotype. Why could pyramiding increase the durability of *R*-genes? The rationale is simple: the pathogen would need double or multiple mutations to overcome the resistance gene. Whether pyramiding would in fact increase durability is however a matter of debate. There are those that have concern about losing two *R*-genes at the same time. Some think that *R*-genes can never be deployed durably in late blight resistance and suggest the use of field resistance again (Fry 2008). In any case, to ensure a maximum lifespan of the late blight *R*-gene, it is important that single-*R*-gene and pyramided plants are not deployed simultaneously. The single-gene plants

would in that case provide a “stepping-stone” for pathogens to overcome each of the pyramided resistance genes (Zhao *et al.* 2003).

Another drawback that is sometimes raised against pyramiding is the possible fitness costs of using extra *R*-genes. *R*-genes may have costs, as was demonstrated in the case of the *Arabidopsis thaliana* bacterial resistance gene *RPM1* (Tian *et al.* 2003). Here, the transformation of plants with RPM1 caused smaller plants, with smaller shoots and lower reproductivity resulting in as much as 9% fewer seeds per plant. The appearance of this article caused much debate about the cost of resistance. A resistance cost of 9% is however so high that it is unlikely that this would be typical for gene-for-gene resistances. *R*-genes are widely used in plant breeding, but an *R*-gene with such high costs would have been quickly eliminated from the breeding pool (Brown 2003).

The mapping and pyramiding of nematode resistance genes

In nematode resistance, the value of *R*-genes has never been contested. *R*-genes like *H1* against *G. rostochiensis* and *Gpa5* against *G. pallida* have already proven their worth. The resistance abilities of major *R*-genes are appealing, especially if they confer absolute levels of resistance.

In this thesis the genetic mapping is described of $GpaXI^{l}_{tar}$ (Chapter 4). The mapping position coincides with a well known *R*-gene cluster on the long arm of chromosome *11*. We postulate that $GpaXI^{l}_{tar}$ is located in one and the same *R*-gene cluster, which already comprises three nematode resistance genes $R_{Mc1\text{-}blb}$, $R_{Mc1\text{-}fen}$, $R_{Mc1\text{-}hou}$, (Draaistra 2006), as well as several more resistance genes against viruses and fungi (Gebhardt and Valkonen 2001). From this cluster, the *R*-gene *N* (Whitham *et al.* 1994) has been cloned, suggesting that $GpaXI^{l}_{tar}$ could represent a TIR-NBS-LRR gene with homology to *N*. The additional value of $GpaXI^{l}_{tar}$ is a relative decrease of 95 % in cyst count which is comparable to the strong-effect QTLs *Grp1* (Rouppe van der Voort *et al.* 1998a) and *Gpa5* (Rouppe van der Voort *et al.* 2000). The non-absolute but partial resistance of the QTL involved in potato cyst nematode (PCN) resistance genes can best be explained by the genetic heterogeneity of the *G. pallida* field populations and by “escapers” that are caused by an ineffective resistance mechanism. Escapers are

easily distinguished from genetic heterogeneity, because the frequency of escapers will not rise during continuous propagation on the same host, whereas genetic heterogeneity will change towards increasing virulence. The molecular basis of the phenomenon of escapers could be based on small-effect mutations in the *R*-gene, affecting the recognition of the product of avirulence genes.

Pyramiding nematode resistance genes

Isolates of potato cyst or root knot nematodes (PCN, RKN) are sexually random mating populations of genetically different individuals. The resulting genetic heterogeneity at avirulence loci is the most probable cause of the non-absolute level of nematode resistance phenotypes. *G. pallida* and *M. chitwoodi* populations can be selected very rapidly for a shift in avirulence factors (Janssen *et al.* 1998; Phillips and Blok 2008; Schouten 1997). The selected nematode population will render the matching *R*-gene without value. The study of Phillips and Blok (2008) however also demonstrates that *G. pallida* populations adapted to resistance from *S. tuberosum spp. andigena* CPC2802, have not acquired the capacity to overcome the resistance of *S. vernei* (CPC 2488 and CPC 2487). This is a very nice illustration of the complementary effect of the two resistance sources. Hence this study is an alternative experimental approach to demonstrate the value of pyramiding of nematode resistance genes, where the spectrum of individual *R*-genes is narrower. From the study of Phillips and Blok (2008) it can be predicted that the joint effect of multiple *R*-genes will result in a broader resistance spectrum and thus in a higher level of resistance.

Will pyramiding work for nematode resistance? In spite of these clear examples for PCN, the RKN pyramiding study in this thesis could not demonstrate an additive level of resistance for *M. hapla* nematode resistance. Because the experiment described in Chapter 5 did not show a significant contribution for $R_{Mh\text{-}tar}$, we can not say if pyramiding of $R_{Mh\text{-}tar}$ and $R_{Mh\text{-}chc}A$ gives an extra higher resistance level.

We can however envision two scenario's when pyramiding nematode resistances. Most importantly, pyramiding *R*-genes with different resistance spectra will stop nematodes with different avirulence spectra. Especially for *G. pallida* it is known that the populations are highly heterogeneous (Folkertsma *et al.* 1996). Pyramiding *R*-genes in this scenario would therefore be very likely beneficial in reducing

reproduction. Another aspect is that nematode *R*-genes can be “leaky”, causing the abovementioned “escapers”. We can only speculate if pyramiding *R*-genes will reduce or even totally prevent these escapers. Unlike with late blight, the resistance response against nematode does not likely rely on the speed of recognition and HR. Hyphal growth of late blight enables colonisation of the next cell and then the next cell, if not stopped by HR. Nematodes are sedentary. We can envision that pyramiding *R*-genes against late blight can increase the chances of recognising a *P. infestans* hyphae and thereby recognising a larger proportion of the invading *P. infestans* hyphae. Because of the sedentary nature of nematodes, it is less likely that the speed of recognition will influence the resistance response; the *R*-gene has plenty of time to recognise it. Furthermore, a successful infection depends, in addition to avoiding HR, also on the ability to induce the development of a feeding site in the root. Escapers are thought to develop because of some sort of “failure” of the resistance mechanism. If escapers are caused by mutations in the *R*-gene, similar as described above in partial resistance of late blight genes, then it is likely that pyramiding *R*-genes can complement this “failure” and will result in absolute levels of resistance.

The value of molecular markers for *R*-gene pyramiding

For breeding purposes, when pyramiding *R*-genes in one variety, the presence of multiple *R*-genes cannot be assessed by phenotypic disease assays, unless the different resistance spectra (if any) of the *R*-genes can be used. When *R*-genes that are used for pyramiding have the same resistance spectrum, it is even impossible to use late blight assays to ascertain that both *R*-genes are introgressed. It is therefore necessary that the genetic positions of the used *R*-genes are known, so one can make use of molecular markers flanking the *R*-genes. In Chapter 3, these molecular markers proved an accurate tool to genotype the pyramiding population and assign the genotypes into the four *R*-gene groups. In this chapter, the individuals of the pyramiding population were genotyped with CAPS markers. In Chapter 5, we attempted to pyramid *M. hapla* resistance genes following the same strategy as in Chapter 3. However, we tried to genotype the individuals of this pyramiding population with AFLP markers. In retrospect, genotyping the pyramiding population with the available AFLP markers proved to be problematic. In this study three cases

were observed where the diagnostic marker alleles were observed in both the resistant as well as the susceptible parents. Obviously, the tightly linked AFLP marker allele did not display a unique association with the *R*-allele. The diagnostic value of marker alleles should be tested against unrelated genotypes or different mapping populations than the mapping population in which the markers were developed. This requires a wide panel of potato genotypes representing the breeders' gene pool. The diagnostic value can be improved only if the marker is within a distance where linkage disequilibrium is significant and should exploit DNA polymorphisms that are haplotype specific (Sattarzadeh *et al.* 2006).

Practical issues in development of highly resistant potato cultivars

The newly mapped *R*-genes described in this thesis have a high value for potato breeding. Apart of the evolutionary aspects discussed before there are also practical considerations. $GpaXI^{l}_{tar}$ is a large-effect PCN resistance gene located on chromosome *11*. This is highly advantageous, in view of the currently used nematode resistance genes. Many other resistance genes have been mapped to potato chromosome *4* and *5*, and have been introgressed in cultivars. Sooner or later it will become impossible to add another *R*-gene, without loosing other alleles from that locus, even at the tetraploid level. This can be solved by tedious analysis of recombinants to have more *R*-genes linked in cis. On the other hand it is highly beneficial to have *R*-genes on different chromosomes to allow the breeder more room to design breeding strategies.

Several *R*-genes involved in late blight resistance have been cloned and several more are expected to be cloned in the near future. After cloning, a cassette of *R*-genes can be build and used to generate GMO potato. We expect that this is the only method to combine many strong, weak, new and broken *R*-genes, and to avoid undesirable linkage drag from the wild potato genome. GMO crops however are confronted with a lack of acceptance by the general public in Europe. Public acceptance could be gained if the environmental benefits of GMO pathogen resistance are properly communicated. In addition, these GMO plants could be placed in a different category,

cisgenesis, which is gaining political support. Cisgenesis is a form of genetic modification where the transferred gene is derived from the same species or a sexually compatible relative. Hence a similar result could have been obtained by classical breeding. Technically, cisgenesis resembles transgenesis, although selection markers are prohibited and the gene should be regulated by its own promoter. It is perceived that the risks associated with cisgenesis do not exceed classical breeding (Jacobsen and Schouten 2007). In the near future it is hoped that cisgenic plants can be exempted from the expensive, time consuming regulations that apply for normal GMO, but this would require an amendment of the current European legislation on the introduction of GMO's.

Another practical recommendation follows from the host range of RKN. It was already mentioned that the stronger evolutionary potential of late blight provides more reason to combine late blight *R*-genes as compared to nematode resistance genes. For PCN resistant cultivars only a limited risk is associated with the one-by-one deployment of individual nematode resistance genes. For PCN it has even been proposed to recommend specific cultivars for specific fields depending on the nematode population ("areaal gebonden teeltadviezen"). In view of the wide host range of RKN it might be wiser to immediately aim for highly resistant cultivars with multiple resistance genes (either pyramiding via classical breeding or gene-cassettes via genetic modification). A strong reduction of the soil infection will be highly beneficial for other susceptible crops that are included in crop rotation.

At this moment resistance levels of potato cultivars in the "Dutch list of varieties" against various pathogens are described by a quantitative trait value. Often a number from 0-10 is used, with 0 being susceptible and 10 being fully resistant to a certain disease. While it might seem like an easy to use system for potato farmers when choosing a particular potato cultivar, the system also has a serious disadvantage. For example, it is known for certain virus resistance genes that resistance is absolute, and not quantitative. Another example assumes a region of PCN infection. It may be wise not to use a cultivar with the same *R*-gene as the previous time, but an *R*-gene with a different resistant spectrum. This might be equally effective as growing a cultivar with both *R*-genes to prevent selection for virulent nematodes. Therefore, a different system should be considered indicating the *R*-genes. It will allow the farmers to realise that different cultivars may possess the same *R*-gene. Furthermore, farmers

may know which *R*-gene is most effective for their fields. It is conceivable that the commercial breeders will greatly benefit from feedback from farmers addressing their evaluations to genes rather than to cultivars. This gene-specific evaluation might be a neglected aspect of participatory plant breeding.

Finally, a few remarks on future perspectives of crop protection are presented. The value of breeding for resistance has been sufficiently explained, but breeding for yield and quality traits may remain more important. Lack of yield or quality cannot be compensated for with pesticides, but lack of resistance can be compensated. The recent interest in breeding for resistance stems from environmental impact of pesticides and the lack of public acceptance for GMO. Sooner or later the public may realise that GMO is not an environmental threat, but can deliver a sound contribution to a sustainable agriculture. Likewise the development of a new generation of non-persistent, non-toxic pesticides could add to an innovative sustainable agriculture. For the balance between the three breeding goals – yield, quality and resistance – the long-term anticipation on future trends by the breeder is of vital importance.

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Summary

Numerous pathogens can infect potato, but late blight (*Phytophthora infestans* (Mont.) de Bary) and potato cyst nematodes (PCN) *Globodera rostochiensis* and *G. pallida* are most damaging. Several species of root knot nematodes (RKN) are an emerging threat. Breeders have successfully deployed disease resistance genes (*R*-genes) to protect potato from diseases, starting from the first half of the 20th century. DNA markers facilitate the introgression of *R*-genes and enable the pyramiding of multiple *R*-genes in a potato cultivar. Pyramiding may improve the level of resistance, the resistance spectrum and the durability of the resistance.

In this thesis, the experimental work is described in four chapters. Two chapters focus on the identification, mapping and characterisation of late blight and nematode resistance genes. The other two chapters focus on the pyramiding of *R*-genes to achieve something more in terms of resistance than was offered by the individual *R*-genes.

In Chapter 2, a locus involved in late blight resistance, derived from *Solanum microdontum*, was identified and characterised. The resistance is associated with a hypersensitive response and results in a delay of infection of about 1-2 weeks. Both a quantitative as well as a qualitative genetic approach was used, based on data from a field assay. QTL analysis identified a QTL on chromosome *4*. A qualitative genetic analysis resulted in the positioning of this locus on the short arm of chromosome *4*. This position coincides with a conserved *Phytophthora R*-gene cluster which includes *R2*, $R_{2\text{-}like}$, $R_{Pi\text{-}blb3}$ and $R_{Pi\text{-}abpt}$. This strongly suggests that $R_{Pi\text{-}mcd1}$ is the fifth *R*-gene of this NBS-LRR cluster.

In Chapter 3, two resistance genes, $R_{Pi\text{-}mcd1}$ and $R_{Pi\text{-}ber}$, introgressed from the wild tuber bearing potato species *S. microdontum* and *S. berthaultii* were combined in a segregating diploid *S. tuberosum* population. Individual genotypes from this segregating population were classified into four groups by means of flanking molecular markers; carrying no *R*-gene, with only $R_{Pi\text{-}mcd1}$, with only $R_{Pi\text{-}ber}$, and a group with the pyramided $R_{Pi\text{-}mcd1}$ and $R_{Pi\text{-}ber}$. The levels of resistance between the groups were compared in a field experiment in 2007. The group with $R_{Pi\text{-}mcd1}$ showed a significant delay to reach 50% infection of the leaf area of three days. The group

with $R_{Pi\text{-}ber}$ showed a delay of three weeks. The resistance level in the pyramid group suggested an additive effect of $R_{Pi\text{-}mcd1}$ with $R_{Pi\text{-}ber}$. This result suggests that potato breeding can benefit from combining individual *R*-genes.

In Chapter 4, a resistance to *G. pallida* Rookmaker (Pa3), originating from wild species *S. tarijense* was identified by QTL analysis. The resistance could largely be ascribed to one major QTL. $GpaXI^{l}_{tar}$ explained 81.3 % of the phenotypic variance in the disease test and mapped to the long arm of chromosome *11*. Another minor QTL explained 5.3 % of the phenotypic variance and mapped to the long arm of chromosome *9*. Clones containing both QTL showed no lower cyst counts than clones with only $GpaXI^{l}_{tar}$. After Mendelising the phenotypic data, $GpaXI^{l}_{tar}$ could be more precisely mapped near markers GP163 and FEN427 thus anchoring $GpaXI^{l}_{tar}$ to a region with a known *R*-gene cluster containing virus and nematode resistance genes.

In Chapter 5, a study is described that tests if pyramiding of two resistance genes against the root knot nematode *Meloidogyne hapla*, $R_{Mh\text{-}tar}$ and $R_{Mh\text{-}chcA}$, will result in improved, or even an absolute level of resistance. $R_{Mh\text{-}tar}$ and $R_{Mh\text{-}chcA}$, introgressed from the wild tuber bearing potato species *Solanum tarijense* and *S. chacoense* were combined in a segregating diploid *S. tuberosum* population. With the aid of markers, descendants from this segregating population were classified into four groups, carrying no *R*-gene, with only $R_{Mh\text{-}tar}$, with only $R_{Mh\text{-}chcA}$, and a group with the pyramided $R_{Mh\text{-}tar}$ and $R_{Mh\text{-}chcA}$. Upon inoculation with *M. hapla* isolate Bovensmilde, the group containing only $R_{Mh\text{-}chcA}$ showed a decline of 88 % in average developed egg masses compared to the group without $R_{Mh\text{-}chcA}$ and $R_{Mh\text{-}tar}$. The group of genotypes containing only $R_{Mh\text{-}tar}$, but not $R_{Mh\text{-}chcA}$, showed a decline of 55% in developed egg masses compared to the group without $R_{Mh\text{-}chcA}$ and $R_{Mh\text{-}tar}$. The effect of both loci, $R_{Mh\text{-}tar}$ and $R_{Mh\text{-}chcA}$ combined, did not further reduce the number of egg masses compared to the level of $R_{Mh\text{-}chcA}$ alone.

The study presented in this thesis shows that marker assisted selection is a very powerful method and sometimes the only way to screen for the presence of certain genes. It furthermore shows that pyramiding different resistance genes, even with minor effects, can result in plants with an increased level of resistance.

Nederlandse samenvatting

Veel ziektenverwekkers kunnen de aardappel infecteren, maar dé aardappelziekte, veroorzaakt door *Phytophthora infestans* (Mont.) de Bary en aardappelmoeheid, veroorzaakt door de aaltjes *Globodera rostochiensis* en *G. pallida* zijn het meest destructief. Daarnaast zijn recent een aantal soorten wortelknobbelaaltjes ook uitgegroeid tot een bedreiging. Vanaf de eerste helft van de twintigste eeuw hebben veredelaars op succesvolle wijze resistentie genen (*R*-genen) tegen ziektes ingekruist om de aardappel resistent te maken tegen deze ziekten. Met DNA merker technieken is het mogelijk om vast te stellen of planten beschikken over de gewenste erfelijke aanleg. Hierdoor kan het inkruisen van *R*-genen makkelijk gevolgd worden, en ze maken het mogelijk om meerdere *R*-genen in een aardappelras te stapelen. Stapelen van resistentiegenen zou niet alleen het niveau van resistentie kunnen verhogen, maar ook het resistentie spectrum kunnen verbreden en de duurzaamheid van de resistentie kunnen verlengen.

Het experimentele werk in dit proefschrift is beschreven in vier hoofdstukken. Twee hoofdstukken zijn gericht op de identificatie, kartering en de karakterisering van resistentie genen tegen de aardappelziekte en aardappelmoeheid. De andere twee hoofdstukken behandelen het stapelen van *R*-genen om een hoger niveau van resistentie te bereiken dan de individuele *R*-genen zouden kunnen bieden.

In Hoofdstuk 2, wordt een locus betrokken bij resistentie tegen de aardappelziekte, afkomstig van *Solanum microdontum*, geïdentificeerd en gekarakteriseerd. De resistentie is geassocieerd met een overgevoeligheidsreactie, en resulteert in een uitstel van infectie van ongeveer 1-2 weken. Resistentie gegevens die middels een veldproef werden verkregen, zijn geanalyseerd met een kwantitatieve en een kwalitatief genetische methode. De kwantitatief genetische methode (QTL analyse) identificeerde een kwantitatief kenmerk (QTL) op chromosoom *4*. Met de kwalitatieve analyse kon dit locus zeer nauwkeurig gekarteerd worden op de korte arm van chromosoom *4*. Deze positie bleek overeen te komen met een welbekend *Phytophthora R*-gen cluster met de eerder gekarteerde NBS-LRR resistentiegenen *R2*, $R_{2\text{-}like}$, $R_{Pi\text{-}blb3}$ en $R_{Pi\text{-}abpt}$. als leden. Dit suggereert dat $R_{Pi\text{-}mcd1}$ het vijfde *R*-gen van dit NBS-LRR cluster is.

In Hoofdstuk 3 wordt het hiervoor beschreven resistentiegen $R_{Pi\text{-}mcd1}$ gecombineerd met een tweede resistentiegen $R_{Pi\text{-}ber}$, geïntroduceerd vanuit de wilde knoldragende aardappelsoort *S. berthaultii*. Hiertoe werd een splitsende diploïde *S. tuberosum* populatie ontwikkeld met deze twee resistentiegenen. De individuele nakomelingen van de populatie werden door middel van flankerende moleculaire merkers in vier groepen geclassificeerd: een groep zonder *R*-gen, met enkel $R_{Pi\text{-}mcd1}$, met enkel $R_{Pi\text{-}ber}$, en een groep met de gestapelde $R_{Pi\text{-}mcd1}$ en $R_{Pi\text{-}ber}$. Het niveau van resistentie werd tussen de groepen vergeleken in een veldexperiment in 2007. De groep met $R_{Pi\text{-}mcd1}$ liet een significante vertraging van drie dagen zien om 50% infectie van het loof te bereiken. De groep met $R_{Pi\text{-}ber}$ liet een vertraging van drie weken zien. Het resistentie niveau in de gestapelde groep lag nog hoger dan hetgeen met de afzonderlijke resistentiegenen bereikt kon worden. Dit verkregen effect suggereerde een additieve werking van beide resistentiegenen $R_{Pi\text{-}mcd1}$ en $R_{Pi\text{-}ber}$. Dit resultaat illustreert dat de aardappelveredeling voordeel kan behalen met het combineren van individuele *R*-genen.

In Hoofdstuk 4 wordt een resistentie tegen *G. pallida* Rookmaker geïdentificeerd door middel van QTL analyse. Deze resistentie is afkomstig van de wilde aardappelsoort *S. tarijense*. Bedoelde resistentie kon voor het overgrote deel verklaard worden door een locus aan het uiteinde van de lange arm van chromosoom *11*. Deze locus $GpaXI^{l}_{tar}$ genaamd verklaarde 81.3 % van de genotypische variantie in de ziektetoets en is derhalve een “major QTL”. Een tweede locus, een minor QTL, verklaarde 5.3% van de fenotypische variantie en karteerde op de lange arm van chromosoom *9*. In nakomelingen met beide QTLs werden niet minder cysten geobserveerd dan met enkel $GpaXI^{l}_{tar}$. Na Mendelisatie van de fenotypische data kon $GpaXI^{l}_{tar}$ nauwkeuriger gekarteerd worden dicht bij merkers GP163 en FEN427. Hieruit kan geconcludeerd worden dat ook $GpaXI^{l}_{tar}$ onderdeel is van een bekend *R*-gen cluster, waartoe al andere virus en nematode resistenties behoren.

In Hoofdstuk 5 wordt een studie beschreven die onderzoekt of het stapelen van twee resistentiegenen tegen het wortelknobbelaaltje *Meloidogyne hapla*, $R_{Mh\text{-}tar}$ en $R_{Mh\text{-}chc}A$, resulteert in een verhoogd of zelfs absoluut niveau van resistentie. $R_{Mh\text{-}tar}$ en $R_{Mh\text{-}chc}A$, afkomstig van de wilde knoldragende aardappelsoorten *S. tarijense* en *S. chacoense* werden gecombineerd in een splitsende diploïde *S. tuberosum* populatie. Met behulp van moleculaire merkers werden nakomelingen van deze segregerende populatie

ingedeeld in vier groepen, zonder R-gen, met enkel $R_{Mh\text{-}tar}$, met enkel $R_{Mh\text{-}chc}A$, en een groep met de gestapelde $R_{Mh\text{-}tar}$ en $R_{Mh\text{-}chc}A$. Na inoculatie met het *M. hapla* isolaat Bovensmilde vertoonde de groep met enkel $R_{Mh\text{-}chc}A$ een afname van 88% in het gemiddelde aantal ontwikkelde eiproppen vergeleken met de groep zonder $R_{Mh\text{-}chc}A$ en $R_{Mh\text{-}tar}$. De groep met genotypen met alleen $R_{Mh\text{-}tar}$, maar zonder $R_{Mh\text{-}chc}A$, liet een afname van 55% in ontwikkelde eiproppen zien vergeleken met de groep zonder $R_{Mh\text{-}chc}A$ en $R_{Mh\text{-}tar}$. Het effect van beide loci, $R_{Mh\text{-}tar}$ en $R_{Mh\text{-}chc}A$ gecombineerd, reduceerde het aantal gevormde eiproppen niet verder dan het niveau dat al met $R_{Mh\text{-}chc}A$ alleen bereikt werd.

Het onderzoek dat in dit proefschrift beschreven wordt laat zien dat merkergestuurde selectie een krachtige, en soms de enige methode is om de aanwezigheid van bepaalde genen te bepalen. Bovendien laat het zien dat het stapelen van verschillende resistentiegenen, zelfs met een zwak effect, kan resulteren in planten met een verhoogd niveau van resistentie.

Dankwoord

ANOVA… dat is toch een verzekeringsmaatschappij of zo?

Bovenstaande zin is letterlijk door mij uitgesproken tijdens mijn sollicitatiegesprek. Ik wil allereerst Herman bedanken voor het vertrouwen dat hij in mij gesteld heeft door mij aan te nemen. Herman… tsja. Onze samenwerking was apart. Af en toe vielen er woorden. Maar uiteindelijk kwam het na elke confrontatie weer goed. Uren en uren zijn er besteed aan filosoferen over het leven, relaties, godsdienst, ethiek en of vrouwen hun benen moeten scheren of niet. Ik zal deze gesprekken missen. Mijn promotor Richard, meestal zag je me alleen als er problemen waren. Terwijl je zo drukbezet bent kon je nog de tijd nemen om naar me te luisteren. En dan had ik nog een officieuze begeleider: Ronald, je nuchtere instelling bracht tegenwicht in al het gestress van de promotie. Ook dank ik je natuurlijk voor alle ijsjes die je voor me hebt gewonnen bij de tourtoto.

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oren van het hoofd af heb gevraagd met belangrijke en onbelangrijke vragen, diegene met wie ik gezellig heb gebabbeld en diegenen die me peptalks hebben gegeven als ik het niet meer zag zitten met het proefschrift wil ik heel erg bedanken.

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Curriculum Vitae

Mei Yi Adillah Tan was born on the 4th of August 1975 in Delft. In 1993 she obtained her VWO diploma at the Hugo Grotius College in Delft. That year she started her study in Forestry at the Wageningen Agricultural University. She did a first M.Sc. thesis at the department of Wood Science. At the department of Plant Physiology she took her second M.Sc. thesis. There she became fascinated with research and decided to pursue a career in science. Therefore she went for an internship in Plant Genetics at the Weizmann Institute of Science in Israel. After her graduation in 1999 she started a Ph.D. in Molecular Plant Physiology at the University of Utrecht. The study on sugar sensing in plants due to *FructokinaseII* unfortunately did not result in the completion of a thesis. After breaking off this Ph.D. research she did a couple of temporary jobs, but worked mainly as a data typist at the Foreign Police. In 2003 she started a Ph.D. at the laboratory of Plant Breeding at Wageningen University and Research Centre under the supervision of dr. ir. Herman van Eck and prof. dr. Richard Visser. The research on development of molecular markers, resistance genes and pyramiding of resistance genes was financed by a foundation 'Stichting Bevordering Veredelingsonderzoek' (SBV) with currently Averis and HZPC as beneficiaries. The results of the research project are described in this thesis. After the Ph.D.-defence, Adillah will travel around the world in a one year trip.

List of publications

Tan, M. Y. A., Alles, R., Hutten, R. C. B., Visser, R. G. F., and van Eck, H. J. 2008. Pyramiding of *Meloidogyne hapla* resistance genes in potato. Accepted in Potato Research.

Tan, M. Y. A., Hutten, R. C. B., Celis, C., Park, T.-H., Niks, R. E., Visser, R. G. F., and van Eck, H. J. 2008. The $R_{Pi\text{-}mcd1}$ Locus from *Solanum microdontum* Involved in Resistance to *Phytophthora infestans*, Causing a Delay in Infection, Maps on Potato Chromosome *4* in a Cluster of NBS-LRR Genes. Molecular Plant-Microbe Interactions 21:909-918

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Jansen, M. A. K., van den Noort, R. E., Tan, M. Y. A., Prinsen, E., Lagrimini, L. M., and Thorneley, R. N. F. 2001. Phenol-oxidizing peroxidases contribute to the protection of plants from ultraviolet radiation stress. Plant Physiology 126:1012-1023.

Submitted:

Tan, M. Y. A., Hutten, R. C. B., Visser, R. G. F., and van Eck, H. J. 2008. The effect of pyramiding *Phytophthora infestans* resistance genes $R_{Pi\text{-}mcd1}$ and $R_{Pi\text{-}ber}$ in potato. Submitted to Theoretical and Applied Genetics.

Tan, M. Y. A., Park, T.-H., Alles, R., Hutten, R. C. B., , Visser, R. G. F., and van Eck, H. J. 2008. $GpaXI^{l}_{tar}$ originating from *Solanum tarijense* is a major resistance locus to *Globodera pallida* and is localised on chromosome *11* of potato. Submitted to Theoretical and Applied Genetics.

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: **M.Y. Adillah Tan**
Date: **3 September 2008**
Group: **Laboratory of Plant Breeding, Wageningen University**

1) Start-up phase	*date*
► **First presentation of your project**	
Molecular markers and pyramiding of R-genes in potato	Feb 25, 2004
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
► **MSc courses**	
PBR-30306 Breeding for Resistance and Quality	Jun-Jul 2007
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student Day 2004, University of Amsterdam	Jun 03, 2004
EPS PhD student Day 2005, Radboud University Nijmegen	Jun 02, 2005
EPS PhD Student Day 2006, Wageningen University	Sep 19, 2006
► **EPS theme symposia**	
EPS theme 4 Genome Plasticity symposium 2003	Dec 10, 2003
EPS theme 4 Genome Plasticity symposium 2004	Dec 09, 2004
EPS theme 4 Genome Plasticity symposium 2006	Dec 08, 2006
EPS theme 4 Genome Plasticity symposium 2007	Dec 07, 2007
► **NWO Lunteren days and other National Platforms**	
ALW Meeting Lunteren 2004	Apr 05-06,2004
ALW Meeting Lunteren 2005	Apr 04-05, 2005
ALW Meeting Lunteren 2006	Apr 03-04, 2006
ALW Meeting Lunteren 2007	Apr 02-03, 2007
Progress meeting Stichting Bevordering Veredelingsonderzoek (SBV) Wageningen	Jan 21, 2004
Progress meeting Stichting Bevordering Veredelingsonderzoek (SBV) Wageningen	Apr 29, 2004
Progress meeting Stichting Bevordering Veredelingsonderzoek (SBV) Metslawier	Aug 31, 2004
Progress meeting Stichting Bevordering Veredelingsonderzoek (SBV) Wageningen	Mar 09, 2005
Progress meeting Stichting Bevordering Veredelingsonderzoek (SBV) Metslawier	Oct 05, 2005
Progress meeting Stichting Bevordering Veredelingsonderzoek (SBV) Valthermond	Mar 27, 2006
Progress meeting Stichting Bevordering Veredelingsonderzoek (SBV) Wageningen	Dec 03, 2006
Progress meeting Stichting Bevordering Veredelingsonderzoek (SBV) Wageningen	Jul 10, 2007
► **Seminars (series), workshops and symposia**	
The truth of science - conference	Aug 12, 2005
workshop li-cor Odissey	Jun 2005
Plant breeding research day	Sep 27, 2007
► **Seminar plus**	
► **International symposia and congresses**	
Triennial conference of the EAPR Bilbao, Spain	Jul 17-22, 2005
► **Presentations**	
Poster presentation EAPR Bilbao	Jul 19, 2005

	Oral presentation at the SBV meetings	2003-2007
►	**IAB interview**	2005
►	**Excursions**	
	Subtotal Scientific Exposure	*17.3 credits**
3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	PE&RC course Basic Statistics	Jun 12-16, 2006
►	**Journal club**	
	Literature discussion at Plant Breeding group	2003-2007
►	**Individual research training**	
	Subtotal In-Depth Studies	*4.5 credits**
4) Personal development		*date*
►	**Skill training courses**	
	Scientific Writing	Oct 2005
	Project Planning and Time management	Sep-Oct 2004
►	**Organisation of PhD students day, course or conference**	
►	**Membership of Board, Committee or PhD council**	
	Subtotal Personal Development	*3.3 credits**
	TOTAL NUMBER OF CREDIT POINTS*	**32,6**

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

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

Cover art: Remko Zijlstra 2008
Models: Adillah Tan and Nicola
Info R.Zijlstra: ezel1@live.nl

The cover art was inspired by the topic of this thesis. Genetic Mapping and Pyramiding of Resistance Genes in Potato
From the author: M.Y.Adillah Tan

The picture on the front cover page symbolises the need for potato protection against diseases by human intervention.