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MAPPING AND CHARACTERISATION OF QUANTITATIVE TRAIT LOCI CONFERRING NEMATODE RESISTANCE IN *SOLANUM SPEGAZZINII*

MAPPING AND CHARACTERISATION OF QUANTITATIVE TRAIT LOCI CONFERRING NEMATODE RESISTANCE IN *SOLANUM SPEGAZZINII*

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Bibliographic Abstract
This thesis describes the mapping and characterisation of QTLs, in *Solanum spegazzinii*, that are involved in resistance to the potato cyst-nematodes *Globodera rostochiensis* and *G. pallida*. For this purpose an RFLP linkage map of potato is constructed based on the offspring from non-inbred parents. Phenomena like distorted segregation and reduced recombination that are observed during linkage analysis are discussed. Several QTLs are mapped that are involved in resistance to *G. rostochiensis* and one major QTL that is involved in resistance to *G. pallida*. Single point analysis and interval mapping are employed for the localisation of the QTLs. Other quantitative traits like tuber yield and root development are mapped as well. In addition a method is described for the non-radioactive detection of single copy DNA-DNA hybrids.

Key-words: restriction fragment length polymorphism, linkage map, potato, *Solanum spegazzinii*, potato cyst-nematodes, *Globodera rostochiensis*, *Globodera pallida*, resistance, quantitative trait loci.

Stellingen

1. Het gezamelijke gewicht van de cysten aan het wortelstelsel heeft een even goede voorspellende waarde voor de mate van resistentie of gevoeligheid van de plant als het aantal cysten.

2. De volgorde van de loci van een samengestelde kaart kan beinvloed worden door de variatie in de recombinatie frequenties tussen de afzonderlijke kaarten.

3. Het in kaart brengen en karakteriseren van kwantitatieve eigenschappen in een kruisbevruchtend gewas dient in een F1- én in een terugkruisings-populatie te gebeuren.

4. De titel van het artikel van Chetelat en DeVerna in TAG (1991) 82:704-712 is niet in overeenstemming met de resultaten die in dat artikel worden gepresenteerd.

5. Een marker is geen merker.

6. Het begrip duurzame resistentie is niet te hanteren in de veredelingspraktijk.

7. De grootte van de werkkamer zegt niets over de kwaliteit van de onderzoeker.

8. De vergelijking tussen een promotie en een bevalling wordt niet alleen gemaakt omdat beiden zoveel inspanning kosten.

9. Roddelen is sociaal.

10. Bescheidenheid ontsiert de mens.

Stellingen behorende bij het proefschrift getiteld "Mapping and characterisation of quantitative trait loci conferring nematode resistance in *Solanum spegazzinii*", door C.M. Kreike, in het openbaar te verdedigen op woensdag 28 juni 1995, te Wageningen.

CONTENTS

Account

Most of the results presented in this thesis have been published before, or will be published in the near future. The content of this thesis has been based on the following publications:

Kreike CM, De Koning JRA, Krens FA (1990) Non-radioactive detection of single copy DNA-DNA hybrids. Plant Mol Biol Rep 8: 172-179

Kreike CM, De Koning JRA, Vinke JH, Van Ooijen JW, Gebhardt C, Stiekema WJ (1993) Mapping of loci involved in quantitatively inherited resistance to the potato cyst-nematode *Globodera rostochiensis* pathotype Ro1. Theor Appl Genet 87: 464-470

Kreike CM, De Koning JRA, Vinke JH, Van Ooijen JW, Stiekema WJ (1994) Quantitatively inherited resistance to *Globodera pallida* is dominated by one major locus in *Solanum spegazzinii*. Theor Appl Genet 88: 764-769

Kreike CM, Van Ooijen JW, Stiekema WJ (1995) Reduced recombination and distorted segregation in a *Solanum tuberosum* x *S. spegazzinii* hybrid. submitted

Kreike CM, Kok-Westeneng AA, Vinke JH, Stiekema WJ (1995) Mapping of QTLs involved in nematode resistance, tuber yield and root development. submitted

List of abbreviations

Ac	acetate
AFLP	amplified fragment lenght polymorphism/polymorphic DNA
AMPPD	3-(2'-spiroadamantane)-4-methoxy-4-(3"- phosphoryloxy) phenyl -1,2- dioxetane disodium salt
ANOVA	analysis of variance
anti-dig	antibody against digoxygenin
AP	alkaline phosphatase
BC	backcross
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	basepairs
BSA	bulked segregant analysis
CAPS	cleaved amplified polymorphic sequence
cM	centiMorgan
CPRO	Centrum voor plantenveredelings- en reproductie onderzoek
DLO	Dienst landbouwkundig onderzoek
DNA	deoxyribonucleic acid
expl var	explained variance
F1, F2	first and second filial generation
IPCR	inverse PCR
ITAL	Instituut voor de toepassing van atoomenergie in de landbouw
h^2	heritability
NBT	nitroblue tetrazolium salt
NC	number of cysts
NT	number of tubers
MAS	marker assisted selection
MGC	marker-genotype class
PCR	polymerase chain reaction
QTL	quantitative trait loci
R^2	variance explained by QTL
RAPD	random amplified polymorphic DNA
RD	root development
RFLP	restriction fragment length polymorphism
RIL	recombinant inbred line
σ_g^2	genetic variance
σ_e^2	environmental variance
SCAR	sequence characterised amplified regions
SDRF	single dosis restriction fragment
sp	species
ssp	subspecies
STMS	sequence tagged microsattelite site
STS	sequence tagged site
SVP	Stichting voor plantenveredeling
TCW	total cyst weight
TE	Tris-EDTA
TTW	total tuber weight

CHAPTER 1

INTRODUCTION

Quantitative traits

The majority of important traits of agricultural crops are inherited quantitatively. These quantitative traits are characterised by a continuous distribution of the phenotypic value. This variation is generally assumed to be due to the presence of multiple loci, each of them indicated as a quantitative trait locus (QTL), and environmental effects (Johanssen 1909; Nilsson-Ehle 1919; East 1915). A subdiscipline of genetics, quantitative genetics, originated in the early part of this century for dealing with such traits. Quantitative geneticists tried to estimate the approximate number of loci, the average gene action (e.g. dominance, recessiveness), and the degree to which the various QTLs interact with each other and the environment, from the continuous phenotypic distribution of a quantitative trait (Falconer 1960; Mather and Jinks 1971). In spite of the complex statistical procedures that were used, the magnitude of the effect of the individual loci and their gene action could not be determined with great precision.

Thoday (1961) proposed to use single gene markers to map and characterise individual QTLs that control quantitative traits. The single gene markers should be scattered throughout the genome of an organism and the segregation of these markers can subsequently be used to detect and estimate the effect of a linked QTL. The first single gene markers that were applied were morphological markers. Sax reported already in 1923 the association between a monogenic trait, seed-coat pigmentation, and a quantitative trait, seed size, in beans. However, for most organisms only a few morphological markers are known and complete genetic maps comprising these markers are scarce, so additional single gene markers are needed for the analyses of quantitative traits.

Biochemical and molecular markers

Isozyme markers were the first biochemical single gene markers that were employed for genetic analyses of quantitative traits (Edwards et al. 1987; Tanksley et al. 1982). These markers were more abundant than morphological markers, but still their number is limited and there are not enough informative markers to cover an entire genome.

The number of molecular single gene markers that can be produced is virtually inexhaustible. These markers are DNA based and distributed over the entire genome. Other advantages compared to morphological markers are their codominance, phenotypic neutrality and their lack of epistatic and pleiotropic effects. Nowadays, several different types of molecular markers are available for genetic studies like RFLPs, RAPDs, STSs, STMSs and AFLPs.

A method to detect DNA sequence variation was first described by Grodzicker et al. (1974). Botstein et al. (1980) named these molecular markers restriction fragment length polymorphism's (RFLPs). The advantage of working with RFLP markers is that this type of marker is codominant and locus specific and can be used in other genotypes and closely related species as well, like for instance in potato and tomato (Bonierbale et al. 1988), cereals (Hulbert et al. 1990) and conifers (Ahuja et al. 1994).

Random amplified polymorphic DNA or RAPD markers (Williams et al. 1990) were developed after the invention of the polymerase chain reaction (PCR) technique (Saiki et al. 1985; Mullis and Faloona 1987). The RAPD technique is very fast but reproducibility between laboratories can be a problem. Another disadvantage is the dominant and genotype specific character of RAPD markers, e.g. for each new genotype a new assay has to be carried out. The dominance of the markers can be a problem when mapping approaches are undertaken in an F2 population. The low information content of the RAPD markers results in less statistical power for mapping (Säll and Nilsson 1994).

Sequence tagged site (STS) markers can be derived from RFLP or RAPD markers (Olsen et al. 1989) and sequence tagged microsatellite site (STMS) markers from microsatellites (Beckman and Soller 1990). These markers are codominant and the PCR dependent DNA amplification has a good reproducibility. In plants, the STS markers are also referred to as CAPS, cleaved amplified polymorphic sequence (Konieczny et al. 1993) and SCAR, sequence characterised amplified regions (Paran and Michelmore 1993). A disadvantage of the STS and STMS markers is that the costs to develop them are very high.

Recently, a new kind of molecular marker, AFLP (amplified fragment length polymorphism), has been developed by Zabeau and Vos (1992). The AFLP technique is very fast and yields an enormous amount of data in a relatively short time (Zethof et al. 1994).

Linkage maps

A linkage map represents the relative order of genetic markers along a chromosome. Recombination frequencies are used to determine the relative distance between the markers. The first linkage maps in crop plants that contained molecular markers were published for inbreeding species like maize, tomato and lettuce (Helentjaris et al. 1986; Tanksley et al. 1987; Landry et al. 1987, resp.). The parents in these crosses were homozygous and F2 or BC generations were used to perform linkage analysis. For outbreeding species like potato and apple, heterozygous parents are used to obtain segregating populations and mapping can be performed in the F1 offspring of a single pair mating (Gebhardt et al. 1989; Hemmat et al. 1994). Since often both parents are heterozygous at many loci, a linkage map can be constructed of both genitors. These linkage maps can be combined with JoinMap (Stam 1993), a software package which has a built-in facility for the integration of genetic maps. The alignment of the homologous parental chromosomes requires at least two common markers for a correct orientation.

Nowadays detailed RFLP linkage maps are available for most important agricultural crops like maize (Coe et al. 1990), tomato and potato (Tanksley et al. 1992; Gebhardt et al. 1991), lettuce (Kesseli et al. 1990), rice (McCouch et al. 1988, Nagamura et al. 1994), *Brassica* (Slocum et al. 1990), *Arabidopsis* (Hauge et al. 1993), soybean (Tingey et al. 1989), alfalfa (Brummer et al. 1993; Echt et al. 1994), bananas (Fauré et al. 1993), cereals (Chao et al. 1989; Chittenden et al. 1994; Devos et al. 1992; O'Donoughue et al. 1994; Philipp et al. 1994; Xie et al. 1993), trees and conifers (Binelli and Bucci 1994; Broome et al. 1994; Devey et al. 1994; Karjalainen et al. 1994; Nelson et al. 1993) and peanut (Halward et al. 1993). Linkage maps are also in development for citrus (Cheng et al. 1994), cocoa (Lanaud et al. 1994), coffee (Paillard et al. 1994; Lashermes et al. 1994), cassava (Angel et al. 1994) and sunflower (Gentzbittel et al. 1994; Jan et al. 1994)

Mapping of quantitative traits with molecular markers

Several statistical procedures can be followed to asses linkage of a QTL and a marker gene. The simplest way is to analyse the quantitative data using one marker at the time in

a one-way analysis of variance (ANOVA) with marker genotypes as classes. This approach is referred to as single point analysis and does not require a complete linkage map (Keim et al. 1990; Reiter et al. 1991). Disadvantage of this method is that it cannot discriminate between the effect of a putative QTL and the degree of linkage with the marker. Due to recombination events between marker and QTL the magnitude of the effect of the QTL will be underestimated. Nevertheless, when a linkage map is available, a graphical representation of the variance 'explained' by each marker may give a first impression as to the genomic regions with 'QTL activity'.

If a complete linkage map is available, so called interval mapping is a better approach for finding linkage between a QTL and marker genes. Instead of analysing the population one marker at the time, pairs of linked markers are used in the analysis. By considering adjacent marker-flanked intervals, the whole genome map is scanned for the presence of QTLs. The recombination distance between the flanking markers is known and enables not only the estimation of the effect of a putative QTL in between the markers but also the estimation of its most likely position in the interval. Therefore the localisation and estimation of the magnitude of the effect of the QTL can be performed more precisely.

Interval mapping was first performed in tomato (Paterson et al. 1988 and 1991) and has subsequently been used successfully for several quantitative traits in other species as well like maize (Stuber et al. 1992; Azanza et al. 1994; González-de-León et al. 1994), *Vigna* (Fatokun et al. 1992), tomato (De Vicente and Tanksley 1993; Aitken et al. 1994), potato (Bonierbale et al. 1994; Leonards-Schippers et al. 1994), soybean (Lark et al. 1994; Webb et al. 1994; Brummer et al. 1994), mungbean (Young et al. 1993) and rice (Ahn et al. 1993; Yano 1994).

Population size and type (BC or F2) are essential factors in the detection of QTLs with small effects (Van Ooijen 1992). To increase the possibility of detecting a QTL, it is very important to choose parents that are significantly different from each other with respect to the trait of interest. Also, selective genotyping of the population, i.e. only genotyping the individuals at the extreme ends of the distribution, can decrease the amount of molecular analyses without great loss of detection power (Lander and Botstein 1989).

Characterisation of quantitative traits

Next to the localisation of QTLs, also other characteristics of genes involved in quantitative traits can be established. First, there is the magnitude of the phenotypic effect of a QTL. It is important to know whether a quantitative trait is controlled by many QTLs, each with a small effect, or only a few QTLs with large effects. The fraction of the phenotypic variation of the quantitative trait that can be explained with the QTLs that have been mapped, is an indication whether major QTLs that contribute to the trait are still undetected.

Secondly, once a QTL has been mapped, the gene action or gene (allele) doses can be resolved with linked molecular markers. Thus far, mostly F2 populations were used to determine the gene dosis (Edwards et al. 1987; Paterson et al. 1991; Stuber et al. 1992).

Thirdly, also epistasis, the interaction between the different QTLs, can be studied. Usually two-way analysis of variance (two-way ANOVA) is employed to determine epistatic interactions between the loci. A few instances of epistasis between QTLs have been reported, but this phenomenon seems not to be prominent (Edwards et al. 1987; Paterson et al. 1988 and 1991; Lark et al. 1994).

Finally, the influence of the environment on a quantitative trait can be studied. One approach to determine QTL-environment interaction is to grow a mapping population in various environments (locations, years) and to perfrom a QTL search for each environment. (Paterson et al. 1991; Stuber et al. 1992). These studies suggest that QTLs with a large effect will be active in several environments, whereas QTLs with small effects can be environment specific. Hayes et al. (1993) noticed that environmental interactions were expressed as differences in the magnitude of the QTL effect. QTLs can also be mapped in different populations and compared with each other, as has been done in maize (Melchinger et al. 1994).

In the approaches described above, a putative QTL is treated as if it were a known single gene. It should be noted that QTLs as detected with the presently available statistical tools may very well be the result of the joint effect of more than one gene. Up till now no cases have been reported where the one-to-one relation between a QTL and a known gene has been demonstrated.

Mapping and characterisation of quantitative trait loci conferring nematode resistance in *Solanum spegazzinii*

In this thesis I describe the mapping and characterisation of QTLs, in *Solanum spegazzinii*, that are involved in resistance to the potato cyst-nematodes *Globodera rostochiensis* and *G. pallida*.

Potato cyst-nematodes

Potato cyst-nematodes can cause severe damage to the roots of a potato plant which eventually will result in considerable yield losses. Two species of potato cyst-nematodes, i.e. *Globodera rostochiensis* (Woll.) and *G. pallida* (Stone) have been described. These nematodes are believed to have originated in the Andean region of Peru and Bolivia (Evans et al. 1975 and 1977) but are now distributed world-wide. The species can be distinguished morphologically by the colour of the females and the cysts, golden yellow in *G. rostochiensis* and white or cream in *G. pallida*, and by differences in the second-stage juveniles (Stone 1972). Also with protein electrophoretic techniques (Trudgill and Parrott 1972; Bakker et al. 1988), RFLPs (De Jong et al. 1989), species-specific monoclonal antibodies (Schots et al. 1988 and 1989) and species specific repetitive DNA sequences (Stratford et al. 1992), these nematode species can be identified.

At present five pathotypes of *G. rostochiensis* and three of *G. pallida* have been recognised in Europe. An international scheme to classify these pathotypes has been proposed by Kort et al. (1977) and is shown in Table 1. The scheme is based upon the ability or inability of the nematode populations to reproduce on a number of particular potato clones, known as differentials. Each clone possess a different set of resistance genes. The Pf/Pi ratio (Pf= formed number of cysts, Pi= initial number of cysts) is used as a measure for the multiplication rate of the nematode population on a differential. The populations are classified as virulent or avirulent if the Pf/Pi ratio are >1 or ≤ 1, respectively.

Resistance against potato cyst-nematodes in *Solanum* species

The genus *Solanum*, to which the cultivated potato belongs, is extremely diverse, containing about 1000 species. In addition to *S. tuberosum*, some six other cultivated species and over 230 wild species of potato are generally recognized (Hawkes 1990). The tuber-bearing wild species are completely confined to the South-American continent

and many are of considerable interest to potato breeders because of their resistance to pests and pathogens.

Table 1. International scheme for potato cyst-nematode nomenclature. A multiplication ratio on a differential genotype ≤ 1 indicates resistance (-), a multiplication ration > 1 indicates susceptibility (+).

		Ro1	Ro2	Ro3	Ro4	Ro5	Pa1	Pa2	Pa3
Clone	plant resistance code								
S. tuberosum ssp *tuberosum*		+	+	+	+	+	+	+	+
S. tuberosum ssp *andigena* CPC 1673 hybr.	Ro1,4	-	+	+	-	+	+	+	+
S. kurtianum hybr. 60.21.19	Ro1,2	-	-	+	+	+	+	+	+
S. vernei hybr. 58.1642/4	Ro1,2,3	-	-	-	+	+	+	+	+
S. vernei hybr. 62.33.3	Ro1,2,3,4 Pa1,2	-	-	-	+	+	-	-	+
S. vernei hybr. 65.346/19	Ro1,2,3,4,5	-	-	-	-	-	+	+	+
S. multidissectum hybr. P 55/7	Pa1	+	+	+	+	+	-	+	+
S. vernei hybr. 69.1377/94	Ro1,2,3,4,5 Pa1,2,3	-	-	-	-	-	-	-	-

The cultivated potato, *S. tuberosum*, originated as a hybrid between the diploid cultivated *S. stenotomum* and the diploid weed *S. sparsipilum*, subsequently followed by chromosome doubling. This event would have taken place in the Andes and the Andean subspecies, *S. tuberosum* ssp *andigena*, was most likely formed first (Hawkes 1990). *S. tuberosum* ssp *tuberosum* does not posses any resistance to the two species of nematodes. Resistance to these major pests have therefore been searched for in many wild potato species (Dellaert et al. 1987, 1988; Turner 1989; Jackson et al. 1988; Van Soest et al. 1983). The most important resistance sources that are presently used by plant breeders are derived from the diploid wild potato species *S. tuberosum* ssp *andigena*, *S. vernei* and *S. spegazzinii*.

S. tuberosum ssp *andigena* CPC 1673 possesses a qualitative resistance locus (*H1*) that provides complete resistance to pathotypes Ro1 and Ro4 of *G. rostochiensis* (Toxopeus and Huijsman 1953). A gene-for-gene relationship has been found between *H1*, which has been mapped on potato chromosome 5 by Gebhardt et al. (1993), and Pineda et al. (1993), and an avirulence gene of *G. rostochiensis* pathotype Ro1 (Janssen et al. 1991).

S. vernei possesses a quantitative resistance against *G. rostochiensis* pathotype Ro1 (Plaisted et al. 1962) and also against some pathotypes of *G. pallida* (Ross 1986). The resistance against both nematodes is presumably polygenic (Ross 1986).

S. spegazzinii is yet another source of resistance to *G. rostochiensis* pathotype Ro1. Ross (1962) described two independent monogenic dominant resistance genes in the accession EBS510, Fa and Fb. The Fa gene is involved in resistance to *G. rostochiensis* pathotype Ro1 and Ro2 while the Fb gene provides resistance to the pathotypes Ro1 and Ro5 and, in combination with minor genes, also to the pathotypes Ro2, 3 and 4 (Ross 1986). A qualitative resistance locus *Gro1* against *G. rostochiensis* pathotype Ro1 from *S. spegazzinii* has been mapped on chromosome *7* (Barone et al. 1990).

S. spegazzinii BGRC 8218 clone 15 is one of the genotypes studied by Dellaert et al. (1988), which showed resistance to *G. rostochiensis* as well as to *G. pallida* pathotypes. The resistance inherited in a quantitative way and the involvement of several genes conferring resistance to both nematodes species was assumed.

Linkage mapping in potato

Since *Solanum tuberosum* ssp *tuberosum* is a tetraploid species, linkage maps have been made using di(ha)ploid genotypes that were crossed either with wild *Solanum* species (Bonierbale et al. 1988) or with other diploid *S. tuberosum* ssp *tuberosum* genotypes (Gebhardt et al. 1989). Segregation analysis in the latter crosses were possible because the parental genotypes are heterozygous and thus enabled linkage mapping in an F1 population. (Gebhardt et al. 1989). The two above mentioned independent RFLP maps have been aligned (Gebhardt et al. 1991) and at this moment a high density molecular linkage map of potato is available (Tanksley 1992).

Several monogenic resistance genes have been mapped on the potato genome, like resistance against *G. rostochiensis* from *S. spegazzinii* (Barone et al. 1990) and from *S. tuberosum* ssp *andigena* (Gehardt et al. 1993; Pineda et al. 1993), potato virus X (Ritter et al. 1991) and *Phytophthora infestans* (Leonards-Schippers et al. 1992; El-Kharbotly et al. 1994). Other monogenic traits that were mapped in potato are the incompatibility locus, SR1, and purple skin color (Gebhardt et al. 1991). Also quantitative traits have been mapped on the potato chromosomes like earliness (Van den Berg et al. 1993) and resistance against *Phytophthora infestans* (Leonards-Schippers et al. 1994) and insects (Bonierbale et al. 1994).

Also at the CPRO-DLO an RFLP map of potato has been constructed with an additional set of markers (this thesis and Jacobs et al. 1995). Using these markers, loci involved in flower pigmentation, tuber shape and skin colour have been mapped (Van

Eck et al. 1993 and 1994a and b) as well as a monogenic resistance locus against *G. rostochiensis* pathotype Ro1 derived from *S. vernei* (JME Jacobs, pers. comm.)

Outline of this thesis

In this thesis, the inheritance of the quantitative resistance to *G. rostochiensis* and *G. pallida*, derived from *S. spegazzinii* BGRC 8218 clone 15, is studied in detail. For this purpose I have mapped and characterised the QTLs conferring resistance to the potato cyst-nematodes using RFLP markers. As mentioned above, a detailed RFLP linkage map is essential for the localisation of quantitative traits. In Chapter 2 I describe the construction of such a linkage map of potato, with markers derived from a genomic library of *S. spegazzinii*. Phenomena like reduced recombination and segregation distortion which were observed during RFLP analysis are discussed as well.

The mapping of the nematode resistance loci is described in Chapter 3, 4 and 5. In Chapter 3, I describe the mapping of loci conferring resistance to *G. rostochiensis* pathotype Ro1, by means of single point analysis, and in Chapter 4 the same method is used for the localisation of QTLs conferring resistance to *G. pallida* pathotype Pa2 and Pa3. Both analyses were performed in an F1 population (*S. tuberosum* x *S. spegazzinii*). In Chapter 5, I describe the use of a backcross population ((*S. tuberosum* x *S. spegazzinii*) x *S. tuberosum*) to localise other quantitative traits like tuber yield and root development in addition to nematode resistance with interval mapping.

RFLP analysis requires the labelling of DNA with radio-isotopes (^{32}P) or non-radioactive ligands for detection. In Chapter 6 I describe a protocol that can be used for non-radioactive labelling and detection of DNA.

In Chapter 7 I discuss several topics that were not mentioned in the previous chapters like mapping in outbreeding species, polyploid mapping, multiallelism and comparative mapping. Future research strategies are also indicated. Finally, the international scheme that is used to classify several pathotypes within the nematode species *G. rostochiensis* and *G. pallida* is discussed.

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CHAPTER 2

RFLP linkage map of potato; Reduced recombination and distorted segregation in a *Solanum tuberosum* x *S. spegazzinii* hybrid

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ABSTRACT

In this paper we describe the reduced recombination in an interspecific hybrid of *S. tuberosum* x *S. spegazzinii*. For this purpose RFLP maps were made of the interspecific hybrid and its parents. The computer program JoinMap was used to construct and combine the separate linkage maps. Furthermore, a reduced recombination frequency was found in the male linkage map of *S. tuberosum* compared to the female linkage map. In a backcross population of the interspecific hybrid with *S. tuberosum*, distorted segregation was further investigated. Gamete selection was mostly responsible for the observed distortion. A clear selection against homozygous genotypes, indicating zygote selection, was found for markers on chromosome *2, 3* and *4*.

INTRODUCTION

Several papers describe the construction of an RFLP linkage map of potato, reflecting its agronomic and economic importance as a crop (Bonierbale et al. 1988; Gebhardt et al. 1989 and 1991; Tanksley et al. 1992). For all maps constructed so far, diploid Solanum species and di-haploid *S. tuberosum* genotypes were used for linkage analysis. Linkage maps comprising molecular markers are useful for the localisation of genes determining agronomic traits. Important loci involved in resistance against phytopathogens have been located on the linkage map of potato, such as resistance to potato cyst-nematodes (Barone et al. 1990; Pineda et al. 1993; Gebhardt et al. 1993; Kreike et al. 1993 and 1994), potato virus X (Ritter et al. 1991), *Phytophthora infestans* (Leonards-Schippers et al. 1992 and 1994; El-Kharbothly et al. 1994) and insects (Bonierbale et al. 1994).

Linkage maps are constructed on the basis of recombination frequencies between markers. Large differences in recombination frequencies are often observed within

eukaryotic species. Besides random variation, sources of variation in recombination frequency are the environment, genetic background, sex and degree of homology between the chromosomes. The best studied environmental influence on recombination frequency is the temperature during meiosis (Tracey and Dempsey 1981; Gavrilenko 1984). Temperature stress induces a significant increase in recombination rate. Evidence for the influence of the genetic background on recombination frequency has been found in *Hordeum vulgare* (Säll 1990), *Glycine max* (Pfeiffer and Vogt 1990) and *Zea mays* (Fatmi et al. 1993). A major nuclear factor that controls the meiotic recombination frequency during female gametogenesis has been mapped in Petunia (Cornu et al. 1989). In *Neurospora* differences in crossing-over frequency in specific chromosomal regions have been shown to result from regulation by region-specific genes (Catcheside 1977; Perkins and Bojko 1992). Clear differences in recombination frequencies between male and female meiosis have been found in *Arabidopsis* (Vizir and Korol 1990), tomato (De Vicente and Tanksley 1991; Van Ooijen et al. 1994) and man (White et al. 1990). Another factor that can influence recombination is the degree of homology between chromosomes. Gebhardt et al. (1991) observed that linkage maps based on interspecific crosses are shorter than linkage maps based on intraspecific crosses of *Solanum* species, presumably caused by a lower degree of homology between the chromosomes in the interspecific hybrid (Radman and Wagner 1993). In yeast meiosis, a random divergence of 10% completely prevents recombination between entire chromosomes (Resnick et al. 1989).

Another phenomenon reported in several linkage analyses published so far is distorted segregation. This systematic deviation from an equal representation of alleles among functional gametes seems to involve all chromosomes and could be caused by selection processes at the gamete or zygote stage. On chromosome *1* of *Lycopersicon peruvianum* a gametophytic self-incompatibility locus has been found that can account for the distorted segregation of alleles from the male parent (Tanksley and Loaiza-Figueroa 1985). An allele from this gene, *SR*1, has been mapped at the same position in potato (Gebhardt et al. 1991).

In this article we describe the reduced level of recombination found in an interspecific hybrid of the cross *S. tuberosum* x *S. spegazzinii*, and the distorted segregation ratios detected in the backcross of this hybrid with *S. tuberosum*. In addition, we compared the level of the male with that of the female recombination in *S.*

tuberosum, which was used as a pistil parent in the production of the F1 population and as a pollen parent in the backcross population.

MATERIALS AND METHODS

Plant material

For the construction of the segregating populations the following diploid genotypes were used: *S. tuberosum* SH 78-88-1320, *S. spegazzinii* BGRC 8218-15 and an interspecific hybrid of these two genotypes. These genotypes will be referred to as Stub, Sspeg and F1-38. The F1 population was produced with the cross Stub x Sspeg (58 plants). The backcross (BC) population was made with plant number 38 of the F1 as F1-38 x Stub (95 plants). By convention in plant genetics a cross is denoted as female x male.

For the construction of the presented RFLP linkage map additional segregation data from an F1 population (58 plants), derived from the cross *S. tuberosum* MH 73-1-106 x *S. phureja* 81-1886-542, were used

RFLP techniques

DNA was isolated from frozen or fresh leaf material according to Dellaporta et al. (1983) with an additional phenol:chloroform (24:1) extraction. Restriction enzyme digestion of the DNA was done following the manufacturer's (Amersham) recommendations. Four restriction enzymes (*Hin*dIII, *Eco*RI, *Eco*RV, *Dra*I) were used to detect RFLPs within the parents. Southern blotting and non-radioactive hybridisation and detection were performed according to Kreike et al. (1990) with minor modifications. The X-ray films were exposed for one to five hours. For hybridisation with radioactive probes, the probes were labelled with random primed labelling kit from USB. (Pre)hybridisation was carried out overnight in 1M NaCl, 1% SDS with salmon sperm DNA. Subsequently, the filters were washed in 0.5 x SSC, 0.5% SDS and placed on X-ray film for three to seven days.

Solanum spegazzinii RFLP markers

The Ssp markers were selected randomly from a genomic DNA library of Sspeg. The library was constructed as follows: total DNA of Sspeg was partially digested with *Pst*I and the resulting fragments were separated on a 1% agarose gel. Fragments of 500 to 3,000 bp were excised, isolated and ligated into the *Pst*I-digested vector pGEM5zf and

transformed to *E. coli* JM101. The clones that were obtained were stored in 15% glycerol at -80°C.

Additional markers

Other RFLP markers that were used in the experiment were: 1) TG clones (tomato genomic clones from SD Tanksley, Cornell University, NY), mapped in potato by Bonierbale et al. (1988) and Gebhardt et al. (1991). We used these markers to identify and orientate the linkage groups. 2) ST and Ac clones, which are genomic clones of *S. tuberosum* ssp *tuberosum* obtained from JME Jacobs (CPRO-DLO). 3) GP clones selected from a genomic library of potato (Gebhardt et al. 1989). 4) cDNA clones encoding proteinase inhibitors PI-1 to PI-4 (Stiekema et al. 1988). Data from two isozyme markers, SKDH1 and PGM2, that segregated in the F1 progeny were kindly supplied by H Bastiaanssen (Wageningen Agricultural University). The segregation of the morphological markers for pigmentation (*P*-locus) and tuber shape (*Ro*) previously described by Van Eck et al. (1993 and 1994, respectively) could be followed in the BC population and these markers were also included in the mapping analysis.

Linkage analyses

The segregation of paternal and maternal alleles was scored in the F1 and BC progeny and four parental linkage maps were constructed. In the crosses four different types of segregation were observed; 1) a two allele backcross type segregation (aa x ab, ab x aa); 2) a two allele F2 type segregation with dominant or codominant alleles (a- x a-, ab x ab); 3) a three allele type segregation (ab x ac); 4) a four allele type segregation (ab x cd).

For the construction of the parental maps only type 1 and type 2 segregation data could be used, therefore, the type 3 and type 4 segregation data were converted into two backcross type segregations (type 1). The construction of the parental linkage maps and the integration of these maps into the presented RFLP map were performed with the package JoinMap (Stam 1993) and Kosambi's mapping function was used (Kosambi 1944). Loci that were shared between the homologous parental chromosomes were used as bridges to align and combine the maps. At least two locus bridges are needed for a correct orientation of the chromosomes.

Comparison of recombination frequencies

For the comparison of levels of recombination, the distances over identical regions on the linkage maps were used. The influence of sex on recombination was assessed by comparing the female and male linkage maps of Stub in the F1 and the BC population, respectively. To inspect the influence of interspecific hybridity without the possibly confounding effects of sex, the (female) linkage map of F1-38 from the BC population was compared to the (female) map of Stub from the F1.

Segregation distortion

The segregation data that were used for the construction of the parental maps (markers of types 1 and 2, and those converted from types 3 or 4) were tested for segregation distortion with a chi-square test for goodness of fit to appropriate expected segregation ratios (1:1, 1:2:1, or 3:1), at a significantce level of P=0.05.

For three loci, Ssp38, PI-4 and Ssp47, a chi-square test (P=0.05) for independence for a 2x2 contingency table was carried out to examine selection at the zygote stage.

RESULTS

Construction of the RFLP linkage map

The genotypes that were used to produce the F1 and BC populations are given in Table 1, together with the direction of the crosses and the number of RFLP markers that segregated for each parent in the progeny. The combined linkage map is shown in Figure 1 and comprises 29 TG, 50 Ssp, 1 ST, 5 Ac, and 2 GP RFLP markers. In addition, we also mapped four cDNAs encoding proteinase inhibitors. Two of them, PI-2 and PI-4, that belong to two different gene families (Stiekema et al. 1988), were mapped to the same position on chromosome *3*; PI-1 was mapped on chromosome *9* and PI-3 on chromosome *7*. The two morphological markers, tuber shape (*Ro*-locus) and pigmentation (*P*-locus) were located on chromosomes *10* and *11*, respectively, and the isozyme loci, SKDH1 and PGM2 on chromosome *1* and *4*. One TG clone, six Ssp clones, one ST clone and one GP clone detected two distinct loci each (indicated with a and b in Figure 1). Three loci were detected with Ssp45 (indicated with a, b and c). In total the map is comprised of 106 loci. The total length of our linkage map is 731 cM (Figure 1) which is of the same order of magnitude as previously published RFLP maps of potato (Bonierbale et al. 1988; Gebhardt et al. 1991; Tanksley et al. 1992).

The positions of the morphological and isozyme markers are in agreement with previously published results (Bonierbale et al. 1988; Van Eck et al. 1993 and 1994). Also no differences were found in the relative positions of most of the TG markers compared to the maps described by Bonierbale et al. (1988) and Tanksley et al. (1992).

Table 1. Number of RFLP markers segregating in each parent (column 4), percentage of markers that showed a distorted segregation (at P= 0.05) (column 5) and the chromosomes involved in the distorted segregation (column 6).

Genotype	Parent	Cross	Number of markers	Segregation distortion	Chromosomes
Sspeg	male	F1	29	24%	*3,5,6,10,12*
Stub	female	F1	41	20%	*1,2,5,7,12*
Stub	male	BC	40	44%	*1,2,3,5,10,12*
F1-38	female	BC	62	35%	*1,2,3,4,5,8,9,11*

Recombination and interspecific hybridity

The recombination frequency of overlapping sections in the RFLP maps of the interspecific hybrid F1-38 and Stub are given in Table 2. The RFLP map of *S. spegazzinii* comprised too few markers and hence too few overlapping segments to include in this analysis. In comparison with Stub, ten out of the fourteen segments are shorter in the interspecific hybrid and on average the segments from Stub are 43% longer.

Figure 1. Linkage map of the potato genome. All loci were mapped with a LOD>3, except for the markers in small print (2<LOD<3) and the markers in parenthesis (LOD<2). The letters (a, b, c) behind the markername indicate that more than one locus is detected with the same probe. For the underlined markers no homozygous genotypes were found in the BC population. The order of markers TG34 and TG48 on chromosome *2* could not be determined unambiguously due to insignificant recombination frequencies but the presented map accords to the orientation in the potato RFLP map of Tanksley et al. (1992). The recombination frequencies of the three markers on chromosome *6* were not significant, however, JoinMap indicated this to be the most likely map, which is in agreement with the other published maps of potato (Bonierbale et al. 1988; Tanksley et al. 1992).

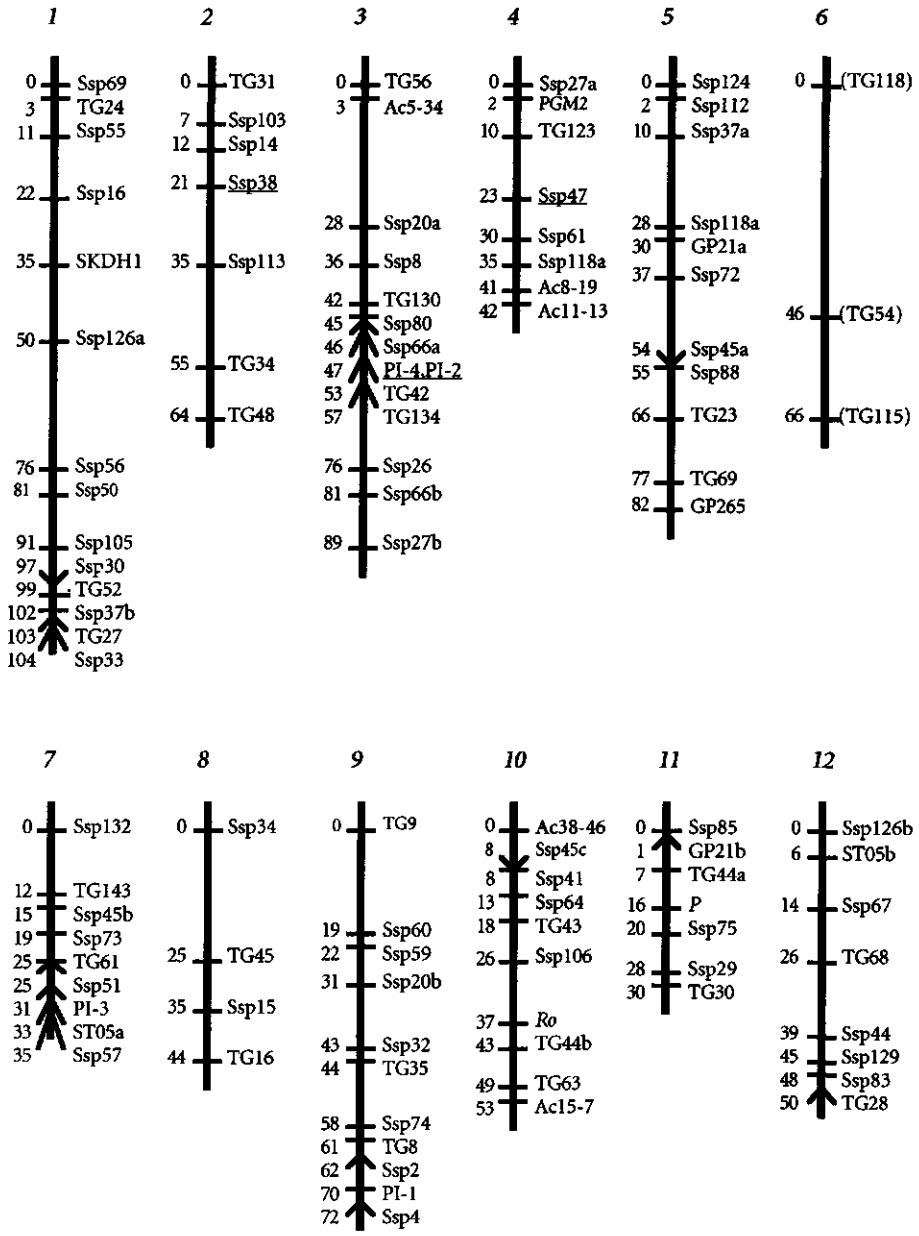

1
0 Ssp69
3 TG24
11 Ssp55
22 Ssp16
35 SKDH1
50 Ssp126a
76 Ssp56
81 Ssp50
91 Ssp105
97 Ssp30
99 TG52
102 Ssp37b
103 TG27
104 Ssp33
2
0 TG31
7 Ssp103
12 Ssp14
21 Ssp38
35 Ssp113
55 TG34
64 TG48
3
0 TG56
3 Ac5-34
28 Ssp20a
36 Ssp8
42 TG130
45 Ssp80
46 Ssp66a
47 PI-4,PI-2
53 TG42
57 TG134
76 Ssp26
81 Ssp66b
89 Ssp27b
4
0 Ssp27a
2 PGM2
10 TG123
23 Ssp47
30 Ssp61
35 Ssp118a
41 Ac8-19
42 Ac11-13
5
0 Ssp124
2 Ssp112
10 Ssp37a
28 Ssp118a
30 GP21a
37 Ssp72
54 Ssp45a
55 Ssp88
66 TG23
77 TG69
82 GP265
6
0 (TG118)
46 (TG54)
66 (TG115)
7
0 Ssp132
12 TG143
15 Ssp45b
19 Ssp73
25 TG61
25 Ssp51
31 PI-3
33 ST05a
35 Ssp57
8
0 Ssp34
25 TG45
35 Ssp15
44 TG16
9
0 TG9
19 Ssp60
22 Ssp59
31 Ssp20b
43 Ssp32
44 TG35
58 Ssp74
61 TG8
62 Ssp2
70 PI-1
72 Ssp4
10
0 Ac38-46
8 Ssp45c
8 Ssp41
13 Ssp64
18 TG43
26 Ssp106
37 Ro
43 TG44b
49 TG63
53 Ac15-7
11
0 Ssp85
1 GP21b
7 TG44a
16 P
20 Ssp75
28 Ssp29
30 TG30
12
0 Ssp126b
6 ST05b
14 Ssp67
26 TG68
39 Ssp44
45 Ssp129
48 Ssp83
50 TG28

Table 2. Comparison between RFLP map of the interspecific hybrid F1-38 and Stub. Both genotypes were used as a female parent. Distance are given in cM.

Chromosome	Segment	F1-38	Stub
1	Ssp55-Ssp16	10.0	22.6
	Ssp16-Ssp126a	26.8	65.2
	Ssp126a-Ssp56	32.7	29.3
	Ssp56-Ssp30	32.5	24.7
2	Ssp103-Ssp38	7.4	10.4
3	TG56-TG130	46.8	92.8
5	Ssp72-Ssp88	15.4	16.3
	Ssp88-TG69	20.2	56.5
7	TG143-TG61	12.5	23.9
	TG61-Ssp57	7.5	12.8
8	Ssp34-TG16	59.4	29.1
9	Ssp59-Ssp32	21.3	12.5
10	Ac46-Ssp106	19.7	50.9
12	TG68-Ssp129	18.4	24.7
	Total	330.6	471.7

Recombination in male and female meiosis

Genotype Stub was used in the F1 cross as a female and in the backcross as a male parent. In both cases a linkage map was constructed for this genotype enabling us to compare the recombination rate in male and female meiosis. The map length of the male and female Stub parent could be compared in eighteen segments distributed over nine chromosomes of which thirteen segments showed a lower male recombination frequency (Table 3). Overall, the female linkage map in these segments was 37% longer than the male linkage map.

Segregation distortion

The segregation of the parental alleles at a given locus was considered distorted if a significant deviation (P=0.05) from an equal representation of those alleles in the offspring was observed. The percentage of segregating markers with a distorted segregation are given in Table 1. All chromosomes appeared to be affected by distorted segregation. No specific chromosomal regions could be identified across all parents which were more involved in this phenomenon than others. Segregation distortion seems to be more frequent in the male parents than in the female parents of a cross (Table 1).

Table 3. Comparison between male and female RFLP map of Stub. Distances are given in cM.

Chromosome	Segment	Stub male	Stub female
1	Ssp55-Ssp16	7.8	22.6
	Ssp16-Ssp126a	36.0	65.2
	Ssp126a-Ssp56	21.2	29.3
	Ssp56-Ssp105	14.3	16.8
	Ssp105-Ssp30	5.0	9.6
2	Ssp103-Ssp38	39.5	10.4
3	TG56-TG130	36.4	92.8
5	Ssp37-Ssp72	17.3	30.1
	Ssp72-Ssp88	17.3	16.3
	Ssp88-TG69	18.9	56.5
7	TG143-TG61	6.7	23.9
	TG61-Ssp57	7.5	12.8
8	Ssp34-TG16	59.4	29.1
9	Ssp59-Ssp32	21.3	12.5
	Ssp32-Ssp20b	14.3	28.9
	Ssp20b-Ssp59	12.4	20.1
11	Ssp85-Ssp75	25.4	23.5
12	TG68-Ssp129	22.2	24.7
	Total	382.9	525.1

In the backcross population, often three different alleles could be monitored at a locus: a *S. spegazzinii* derived allele from F1-38 (speg-allele), a *S. tuberosum* allele shared by F1-38 and Stub (tub1-allele), and a unique *S. tuberosum* allele derived from Stub (tub2-allele). The segregation of these alleles was analysed in more detail (Figure 2). More speg-alleles were found along chromosomes *1*, *3*, *4*, *5* and *8*, while a shortage of speg-alleles was found along chromosome *11*. Overall, the total percentage of speg-alleles in the BC population was 54.1%. The segregation of the tub2-allele, derived from Stub is also presented in Figure 2. More tub2-alleles were found along chromosome *2*, *3*, 5, *7*, *9* (*7* and *9* showed no significant segregation distortion, however). While a shortage of the tub2-allele was found along chromosome *4* and *12*.

To examine selection at the zygote stage, loci displaying a type 3 segregation in the BC population were used (Figure 2). Normally the assortment of gametes is assumed to be independent of the genotype. Accordingly, the expected fraction of homozygous genotypes (tub1/tub1) was calculated on the basis of the parental segregation ratios. In general the observed fractions of homozygous genotypes were close to the expected values, but for markers Ssp38, PI-2/PI-4 and Ssp47 (on chromosomes 2, 3 and 4 respectively) The observed frequencies were extremely low. The segregation of these

markers is shown in more detail in Table 4, which revealed a very significant deviation from an independent assortment.

Table 4. Observed and expected segregation ratios in the BC population for the loci Ssp38, PI-4 and Ssp47. The chi-square test for independance for a 2x2 contingency table was highly significant (P<0.001) for all three markers.

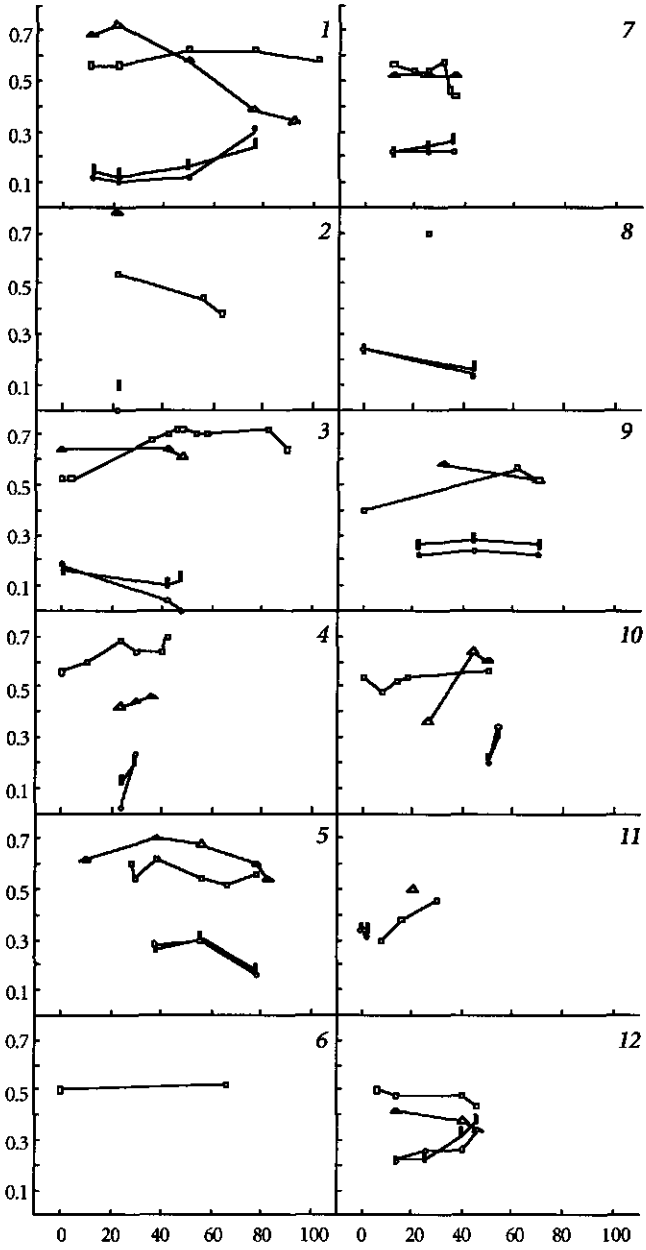
	Stub	tub1		tub2		
Marker	F1-38	tub1	speg	tub1	speg	chi-square
Ssp38	observed	0	17	40	33	16.8
	expected	8	9	32	41	
PI-4	observed	0	33	26	27	23.2
	expected	10	23	16	37	
Ssp47	observed	1	32	24	22	21.5
	expected	10	23	15	31	

DISCUSSION

Construction of the RFLP linkage map

The computer program JoinMap (Stam 1993) was used to construct the presented linkage map of potato. The program combines the recombination frequency data of the segregating loci of the various parents. This has to be performed carefully, as the recombination frequencies are influenced by, for instance, sex and hybridity. If the parents share only a few segregating markers for a chromosome, the distance between these markers will be averaged, whereas the recombination values with the other segregating markers will retain their (non-averaged) parental values. Due to these calculations the order of the markers on the integrated map may change compared to the order of the separate parental maps. These differences between the separate maps and the integrated map have to be analysed in detail and if necessary, fixed sequences must be imposed on the JoinMap calculations to keep the markers in the correct order. The main purpose of the construction of an integrated linkage map is to find the most likely order of the markers along a chromosome. The calculated distances between the markers become less meaningfull.

Figure 2. Fraction of the speg-allele derived from F1-38 (-Δ-) and the tub2-allele derived from Stub (- -) in the BC population. The expected frequency for both alleles is 0.5. The observed (-O-) and expected (-I-) fraction of homozygous genotypes in the BC population are also shown. On the x-axis the position of the markers on the chromosome is given in cM, these positions correspond to the positions in figure 1.


1
2
3
4
5
6
7
8
9
10
11
12
0.7
0.5
0.3
0.1
0
20
40
60
80
100

Recombination and interspecific hybridity

We compared the female linkage map of the interspecific hybrid F1-38 with the female Stub map (Table 2) and observed a tendency for a reduced recombination frequency in the interspecific hybrid. Comparison of other linkage maps of potato also indicates a reduced recombination frequency in interspecific hybrids. The linkage maps published by Bonierbale et al. (1988) and Tanksley et al. (1992), based on interspecific hybrids, are consideraly shorter than the linkage map of Gebhardt et al. (1991) which is constructed with pure *S. tuberosum* genotypes.

A cause for the reduced recombination frequency in the interspecific hybrid might be the abortion of those gametes which received a high number of recombinant chromosomes (Rick 1969) or the elimination of highly recombinant zygotes (Gadish and Zamir 1987). Bonierbale et al. (1988) and Gebhardt et al. (1991) noted that regions with low recombination frequencies also exhibited distorted segregation ratios. For Table 2 only three segments could be identified where distorted segregation at one or two loci might be responsible for the observed shorter map length. Theoretical studies performed by Van Ooijen et al. (1994) and Säll and Nilsson (1994) showed that segregation distortion caused by selection at a single locus does not influence the estimation of the recombination frequency to a great extent, whereas selection at two loci can lead to an underestimation of the recombination frequency. Since only three segments contained markers that displayed a distorted segregation, we do not presume that the lower recombination frequencies found in the interspecific hybrid are a consequence of segregation distortion.

A different explanation could be the decrease in homology between the two chromosome sets in the interspecific hybrid. Recombination requires sufficient homology to enable productive DNA interactions. In yeast meiosis, efficient recombination does occur between blocks of 1,000 basepairs of perfect homology embedded in non-homologous regions (Haber et al. 1991), but a random divergence of as little as 10% completely prevents recombination between entire chromosomes (Resnick et al. 1989; Radman and Wagner 1993). Since we found no indication that the reduced recombination in the interspecific hybrid is caused by segregation distortion, we presume that it is caused by a difference in homology between the chromosomes as a consequence of divergent evolution of the two species involved.

Recombination frequency in male and female meiosis

Our findings concerning the male and female map length of *S. tuberosum* (Table 3) show a tendency for a lower recombination frequency in the male parent. Differences in recombination between the male and female parent based on chiasmata frequencies have been reviewed by Burt et al. (1991). More detailed studies on male and female recombination frequencies with molecular markers in *Arabidopsis* (Vizir and Korol 1990), *Lycopersicon* (De Vicente and Tanksley 1991; Van Ooijen et al. 1994) and man (White et al. 1990) showed that the recombination frequency varies between the sexes. Gebhardt et al. (1991) reported no difference between male and female linkage maps of *S. tuberosum.* However, in that study, the male and female linkage maps of two different genotypes were compared and a genotype specific difference in recombination frequency could have masked a possible sex-related variation in recombination frequency.

For Table 4 only five segments, could be identified where distorted segregation at one or two loci might be responsible for the observed shorter map length, therefore we do not presume that the lower recombination frequencies found in the male parent are a consequence of segregation distortion.

Segregation distortion

We studied distorted segregation in the interspecific hybrid F1-38 in detail and observed it on chromosome *1, 2, 3, 4, 5, 8, 9* and *11.* Distorted segregation was also observed in the linkage analyses of Bonierbale et al. (1988) and Gebhardt et al. (1989 and 1991) on the chromosomes *1, 2, 3, 6, 7, 8, 10* and *1, 2, 3, 5, 10, 12,* respectively. Some overlapping regions displaying distorted segregation can be noted across these linkage maps, for instance the region around Ssp16 chromosome *1*, the distal end of chromosome *2* near TG48, the region near Ssp72 on chromosome *5* and around TG63 on chromosome *10*, while other regions seem more cross or genotype specific.

There are several possible causes for the unequal segregation of alleles such as the abortion of male and female gametes or selective exclusion of particular gametic genotypes from fertilisation due to incompatibility, incongruity, certation or zugote selection. The gametophytic self-incompatibility locus on chromosome *1* can cause distorted segregation of markers closely linked to this locus and can explain the observed segregation distortion in the male parent of markers around marker Ssp16.

Incongruity is a non-functioning between two partners which is caused by a lack of genetic information in one parent about structure and physiology of the other parent

(Hogenboom 1973). Pollen without this information is unable to fertilise the eggs. Chetelat and De Verna (1991) found loci on chromosomes *6* and *10* of tomato that could be involved in incongruity. However, in the BC population no distinct segregation distortion was observed at these loci.

Certation is the competition in growth rate between pollen tubes of different genotypes. Zamir and Tadmor (1986) studied the proportion of unequally segregating genes in intra- and interspecific crosses of the genera *Capsicum* and *Lycopersicon*. The direction of the segregation distortion in intraspecific hybrids of *Lycopersicon* back crossed to the cultivated parent was in favour of alleles from the wild parent, while in *Capsicum* the alleles of the cultivated parent were favoured. In the BC population we observed a slight preference for the speg-alleles (54%).

The absence of the tub1/tub1 genotypes on chromosome *2*, *3* and *4* in the backcross population indicates that selection is taking place at the zygote level against homozygous genotypes. A possible explanation for this phenomenon is the close linkage of (sub)lethal or recessive factors to these marker loci. The subsequent elimination of homozygous genotypes at these loci can result in the distorted segregation found in F1-38 and Stub for chromosome *2*, *3* and *4*.

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CHAPTER 3

Mapping of loci involved in quantitatively inherited resistance to the potato cyst-nematode *Globodera rostochiensis* pathotype Ro1

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ABSTRACT

We report the identification and mapping of two quantitative trait loci (QTLs) of *Solanum spegazzinii* BGRC, accession 8218-15, involved in resistance to the potato cyst-nematode *Globodera rostochiensis* pathotype Ro1, by means of restriction fragment length polymorphisms (RFLPs). For this purpose we crossed a susceptible diploid *S. tuberosum* with the resistant *S. spegazzinii*, and tested the F1 population for resistance to the Ro1pathotype. Since the F1 segregated for the resistance, the *S. spegazzinii* parent was concluded to be heterozygous at the nematode resistance loci. For the mapping of the resistance loci we made use of RFLP markers segregating for *S. spegazzinii* alleles in the F1. One hundred and seven RFLP markers were tested in combination with 4 different restriction enzymes; 29 of these displayed a heterozygous RFLP pattern within *S. spegazzinii* and were used for mapping. Analysis of variance (ANOVA) was applied to test the association of the RFLP patterns of these markers with nematode resistance. Two QTLs involved in disease resistance to *Globodera rostochiensis* pathotype Ro1 were identified and mapped on chromosome *10* and *11* respectively.

INTRODUCTION

Potato cyst-nematodes belong to the major pests of potato. Two potato cyst-nematode species are known, *Globodera rostochiensis* (Woll.) and *G. pallida* (Stone), and several pathotypes have been described for each species (Kort et al. 1977). Resistance to Ro1, the most important pathotype of *G. rostochiensis*, has been identified in several *Solanum* species (Ellenby 1952 and 1954). A monogenic resistance locus (*H1*) present in *S. tuberosum* ssp *andigena* (Toxopeus and Huijsman 1953) has been widely used in plant breeding and provides complete resistance to pathotypes Ro1 and Ro4. Recently a gene-for-gene relationship between this resistance gene (*H1*) from *S. tuberosum* ssp *andigena* and the avirulence gene of *G. rostochiensis* has been proven by Janssen et al. (1991).

Another source of resistance to *G. rostochiensis* pathotype Ro1 is *S. spegazzinii*. *S. spegazzinii* is a diploid wild potato species which displays resistance to several *G. rostochiensis* and *G. pallida* pathotypes (Dellaert et al. 1988). Ross (1962) described two independent monogenic dominant resistance genes, *Fa* and *Fb*, in the accession EBS510. The *Fa* gene is involved in resistance to *G. rostochiensis* pathotype Ro1 and Ro2 while the *Fb* gene provides resistance to the Ro1 and Ro5 pathotypes and, in combination with minor genes, also to the Ro2, 3 and 4 pathotypes (Ross 1986).

With the use of molecular markers like RFLPs, linkage maps of the potato chromosomes have been constructed (Bonierbale et al. 1988; Gebhardt et al. 1989 and 1991) that allow the genetic localisation of genes of interest. Concerning nematode resistance in potato, a resistance locus *Gro1* against *G. rostochiensis* pathotype Ro1 from *S. spegazzinii* has been mapped on chromosome *7* (Barone et al. 1990) and the *H1* resistance gene from *S. tuberosum* ssp *andigena* on chromosome 5 (Gebhardt et al. 1993; Pineda et al. 1993).

For the introgression of monogenic traits into *S. tuberosum*, screening and selection for the phenotype can be straightforward, but for quantitative traits, marker-based selection with RFLPs is advantageous. In this paper we describe the inheritance of the quantitative resistance to the *G. rostochiensis* Ro1 pathotype from *S. spegazzinii* BGRC accession 8218-15. The aim of this research is to map the loci involved in this resistance by means of RFLPs. Markers close to the resistance loci can subsequently be used for marker-based selection. To gain further insight into the nature of the detected resistance loci we also analyzed the interaction between the genes and their individual contribution to the resistance.

MATERIALS AND METHODS

Plant material

S. spegazzinii BGRC accession 8218-15 (Sspeg) was used as a pollen donor in a cross with the diploid susceptible *S. tuberosum* SH 78-88-1320 (Stub). Ninety-six F1 plants were tested for resistance to *G. rostochiensis* pathotype Ro1 as described below. The *S. spegazzinii* parent appeared to be heterozygous for the resistance loci because resistance to pathotype Ro1 was segregating in the F1 progeny. Mapping of the resistance genes with RFLPs was, therefore, performed in this F1 population.

RFLP analyses

Thirty-one genomic tomato clones (Tanksley et al. 1987) and 76 genomic *S. spegazzinii* clones, indicated by TG and Ssp, respectively, were used in combination with four restriction enzymes (*Hin*dIII, *Eco*RI, *Eco*RV, *Dra*I) to detect heterozygous RFLP patterns within *S. spegazzinii*. Segregating RFLP patterns in the F1 progeny were required to identify associations with the resistance loci that were also segregating in the F1 population. The chromosomal location of the TG clones in potato has been determined previously (Bonierbale et al. 1988; Gebhardt et al. 1991) and the 31 TG clones we used were evenly distributed over the potato chromosomes. The 76 Ssp clones were a random sample of a genomic *Pst*I library from *S. spegazzinii* 8218-15, and are currently being mapped on the potato chromosomes in another population as well (C.M. Kreike, manuscript in preparation).

Leaf material was harvested during propagation of the plants in the greenhouse and stored at -80°C. DNA isolation and digestion were carried out according to Dellaporta et al. (1983). Southern blotting and hybridisation were performed with the non-radioactive hybridisation and detection techniques described previously (Kreike et al. 1990).

Three consecutive RFLP screenings were carried out. (1) Initially a random sample of 57 F1 plants was taken. (2) If the association between marker-genotype and nematode resistance was significant at the 10 % level an additional set of 19 plants was screened. (3) If association at the 5 % significance level was found, all F1 plants were screened with that RFLP marker. Equal segregation of the RFLP alleles in the F1 population was determined with the chi-square goodness-of-fit test.

Resistance test

Five tubers of each F1 individual were tested for resistance to *G. rostochiensis* pathotype Ro1 in a randomized block experiment. The parents and a susceptible standard (*S. tuberosum* cv Maritta) were included in the test. Cysts from the "Mierenbos A" population of *G. rostochiensis* pathotype Ro1 (Arntzen and van Eeuwijk, 1992) were used as inoculum. Sprouted tubers (one per replicate) were planted in separate clay pots, 10 cm in diameter (295 ml), which were filled with loam sand. The pots were each inoculated with 30 cysts enclosed in a nylon net, allowing the hatched larvae to pass and invade the roots. Growing conditions, soil temperature and soil moisture control were as described by Van der Wal and Vinke (1982). After 4 months of plant growth the water

supply was gradually stopped. One month later, when the soil was air-dry, the cysts were recovered by flotation. The nylon inoculation net was retrieved so that only newly-formed cysts were collected and counted.

Statistical analysis

A normalizing transformation [$^{10}\log(x+1)$] was performed on the counts of the newly-formed cysts per replicate. These transformed data of the resistance test were investigated with analysis of variance (ANOVA) for a randomized block design. From this ANOVA the heritability of the genotypic mean values for Ro1 resistance could be calculated with formula (1):

$$h^2 = \frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_e^2}{5}} \qquad (1)$$

h^2 = heritability, σ_g^2 = genetic variance, σ_e^2 = environmental variance

The association of marker genotypes with resistance was assessed with one-way ANOVAs based on the averages over the five replicates of the log-transformed cyst counts. A significance level of 5% was employed. The magnitude of the marker-associated phenotypic effect is described by the coefficient of determination (R^2), which represents the fraction of the total variance accounted for by the marker genotypes.

The interaction between the loci involved in Ro1 resistance was resolved by constructing a two-way ANOVA. The RFLP patterns of two markers were combined so that four marker-genotype classes were obtained. The mean number of cysts was calculated for each class.

RESULTS

RFLP analysis

Two different sets of markers, TG and Ssp, were employed to detect RFLP patterns that segregated for *S. spegazzinii* alleles in the F1 population. This screening yielded 29 suitable clone-restriction enzyme combinations (Table 1). None of the four markers

known to map on chromosome *8*, showed segregation for *S. spegazzinii* alleles. The chromosomal location of the 29 clones is given in Table 1, column 1, while one Ssp marker could not be mapped on the chromosomes. The localisation of the Ssp markers on the chromosomes was done using another population as well (C.M. Kreike, manuscript in preparation). Most loci followed a 1 : 1 segregation pattern but seven out of the 29 clones (24%) showed distorted segregation (Table 1, column 2). Only 27% of the markers that were used to find heterozygous *S. spegazzinii* alleles was informative, whereas 40% were heterozygous for *S. tuberosum* alleles.

Table 1. Segregation ratios of RFLP markers heterozygous for *S. spegazzinii* alleles and the association of these markers with nematode resistance against *G. rostochiensis* pathotype Ro1.

Marker	Chromosome	Ratio	P
Ssp55	*1*	30 : 44	
TG34	*2*	25 : 28	
Ssp80	*3*	32 : 27	
TG134	*3*	17 : 32*	
Ssp27	*4*	47 : 40	(0.053)
TG123	*4*	37 : 32	
Ssp124	*5*	29 : 22	
Ssp112	*5*	37 : 30	
Ssp37	*5*	36 : 33	
Ssp72	*5*	33 : 21	
TG23	*5*	30 : 11*	
TG118	*6*	20 : 53*	
TG115	*6*	37 : 37	
Ssp132	*7*	27 : 26	
TG143	*7*	48 : 33	
Ssp51	*7*	39 : 31	
Ssp57	*7*	25 : 19	
TG8	*8*	38 : 28	
TG9	*8*	36 : 19*	
Ssp64	*10*	49 : 42	
TG63	*10*	53 : 31*	0.014
Ssp75	*11*	43 : 50	0.012
TG30	*11*	29 : 40	0.041
TG28	*12*	19 : 31	
Ssp83	*12*	14 : 36*	
Ssp129	*12*	18 : 24	
Ssp67	*12*	25 : 26	
Ssp126b	*12*	25 : 23	
Ssp45		15 : 36*	

* significant distortion of the segregation ratio 1:1 ($P < 0.05$)

P = probability level for association with disease resistance, only shown if $P < 0.05$.

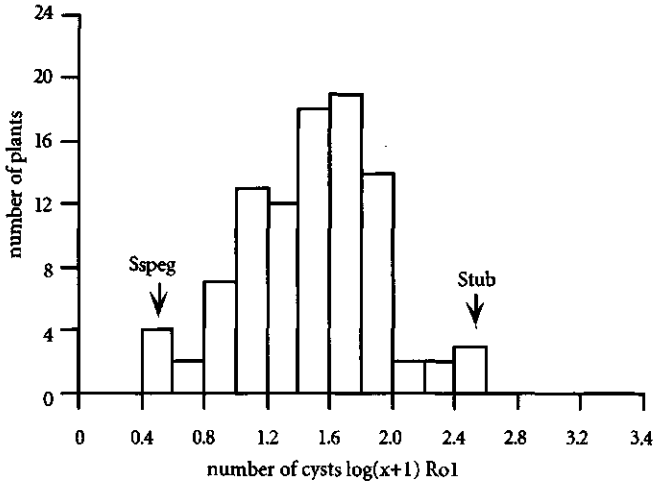

Figure 1. Frequency distribution of the mean number of cysts formed on the 96 F1 plants.

Statistical analysis

The number of newly-formed cysts in the F1 population ranged from zero to almost 500. One cyst and 323 cysts were formed respectively on the resistant *S. spegazzinii* parent and the susceptible *S. tuberosum* parent while on the susceptible standard cultivar Maritta, approximately 1300 cysts were counted. The frequency distribution of the average of the log-transformed cyst count per F1 genotype is given in Fig. 1. Kurtosis and skewness were 0.006 and -0.093, respectively, which is not indicative of a significant deviation from a normal distribution. This means that the Rol resistance studied is quantitatively inherited . The genetic variance was very significant ($P<0.001$, Table 2) and emphasised the segregation of resistance loci in the F1. The heritability of the genotypic mean values for Rol resistance was estimated to be 0.63.

Table 2. Analysis of variance (ANOVA) of the five replications after transformation [$^{10}\log(x+1)$] of the original data of the resistance test.

Source	df	SS	MS	P
Between replications	4	5.37	1.34	0.002
Between F1 plants	95	80.00	0.84	<.001
Residual	351	107.50	0.31	

One-way ANOVAs were carried out to find associations between the 29 RFLP markers which showed segregation for the *S. spegazzinii* alleles and the nematode resistance of the F1 progeny. *P*-values <0.05, with respect to the association of the RFLP markers with Ro1 resistance, are indicated in Table 1, column 4. The only three RFLP markers that fulfil this requirement are TG63 on chromosome *10* and Ssp75 and TG30 on chromosome *11*. Ssp75 and TG30 were linked by 10 cM (Figure 2). The map position of Ssp75 was also confirmed by placing it within the framework of the potato RFLP map of Gebhardt et al. (1991). The marker Ssp27 on chromosome 4 is on the verge of significance (P= 0.053). Table 3 (a, b) shows the ANOVA results for the markers TG63 and Ssp75; the latter has a higher significance level for being associated with an Ro1 resistance locus on chromosome *11* than does TG30. These data indicate that loci are present on the chromosomes *10* and *11* which are involved in quantitative resistance to *G. rostochiensis* pathotype Ro1.

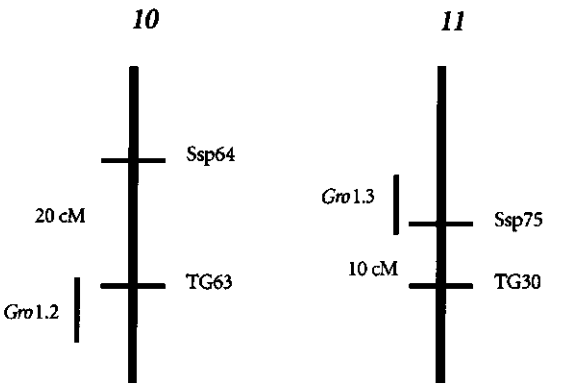

Figure 2. Localisation of the resistance loci Gro1.2 and Gro1.3 on chromosome 10 and 11, respectively.

Since the ANOVA assumes a normal distribution of the data, and this assumption might be violated with the present data, we verified the ANOVA results by performing a non-parametric test (Mann-Whitney U-test) on the 29 RFLP markers. Again the markers TG63, Ssp75 and TG30 were the only ones to be significantly associated (P< 0.05) with Ro1 resistance (data not shown). These results are again a strong indication

that loci are present on chromosomes *10* and *11* which are involved in quantitative resistance to *G. rostochiensis* pathotype Ro1.

Table 3a. One way ANOVA for association of the RFLP marker TG63 with disease resistance against *G. rostochiensis* pathotype Ro1. Below the ANOVA the mean number of cysts calculated for each marker-genotype class are presented. MGC= marker-genotype class.

TG63

Source	df	SS	MS	F value	P
Between MGC	1	1.1	1.1	6.34	0.014
Within MGC	82	14.21	0.17		
Total	83	15.31			

MGC	Number of plants	Mean	Std. Error
1	53	1.56	0.06
2	31	1.32	0.07

Table 3b. One way ANOVA for association of the RFLP marker Ssp75 with disease resistance against *G. rostochiensis* pathotype Ro1. Below the ANOVA the mean number of cysts calculated for each marker-genotype class are presented. *MGC= marker-genotype class.*

Ssp75

Source	df	SS	MS	F value	P
Between MGC	1	1.06	1.06	6.56	0.012
Within MGC	91	14.72	0.16		
Total	92	15.78			

MGC	Number of plants	Mean	Std. Error
1	43	1.60	0.06
2	50	1.39	0.06

The segregation of RFLP markers showing heterozygosity of the susceptible parent *S. tuberosum* SH 78-88-1320 was also scored in the F1 population. Ssp75 which, was associated with nematode resistance in *S. spegazzinii*, also segregated for *S. tuberosum* alleles. Forty-three RFLP markers were examined with ANOVA for associations with Ro1 resistance as described above (data not shown). None of the 43 RFLP markers were significantly associated with Ro1 resistance; therefore, no influence on the resistance from the susceptible parent *S. tuberosum* could be detected.

The coefficient of determination (R^2) calculated for TG63 and Ssp75 was 7% for both. This means that 14% of the total variation and 22% of the genetic variation can be explained by these two markers.

To determine the interaction between the two RFLP loci that were associated with resistance to *G. rostochiensis* pathotype Ro1, a two-way ANOVA was performed (Table 4). No interaction was found between markers TG63 and Ssp75 indicating that both loci were additive.

Table 4. Two-way ANOVA table of the markers TG63 and Ssp75. Below the ANOVA the mean number of cysts calculated and the number of plants (in brackets) for each marker-genotype class are given.

Source	df	SS	MS	F value	P
TG63 (A)	1	1.21	1.21	7.76	0.007
Ssp75 (B)	1	1.34	1.34	8.60	0.004
Interaction	1	0.40	0.40	2.60	0.111
Error	79	12.29	0.16		

		Ssp75 1	2	total
TG63	1	1.65 (21)	1.50 (32)	1.56 (53)
	2	1.52 (16)	1.07 (14)	1.31 (30)
	total	1.59 (37)	1.37 (46)	1.47 (83)

DISCUSSION

Genetic diversity

Ross (1962) was the first who described the inheritance of the resistance of *S. spegazzinii* EBS 510 to *G. rostochiensis*. He reported high numbers of resistant progeny after crossing *S. spegazzinii* with a susceptible *S. tuberosum* and postulated that two independant dominant genes were responsible for the resistance. Momeni et al. (1969) used another genotype of *S. spegazzinii* and detected only one dominant gene involved in nematode resistance. Also Barone et al. (1990) found a clear 1:1 segregation in an F1 population, indicating monogenic inheritance of this resistance trait. The present study, however, employed yet another source of *S. spegazzinii* which does not reveal a simple qualitative inheritance of the resistance to pathotype Ro1, but rather a quantitative

inheritance. These data show that the species *S. spegazzinii* is very diverse with respect to nematode resistance as was already noted by Jones and Pawelska (1963).

Furthermore, the loci involved in quantitative Ro1 resistance described here, are located on different chromosomes to the locus mapped by Barone et al. (1990), who located the monogenic resistance *Gro1* on chromosome *7*. They are also different from the resistance locus *H1* from *S. tuberosum* ssp *andigena*. This locus is mapped to chromosome *5* (Gebhardt et al. 1993; Pineda et al. 1993). Quantitative resistance to *G. rostochiensis* pathotype Ro1 has only been previously observed in *S. vernei* (Plaisted et al. 1962). We describe here for the first time quantitatively-inherited resistance to *G. rostochiensis* pathotype Ro1 from *S. spegazzinii*. We were able to observe and map this quantitative resistance since no monogenic resistance locus was present in our *S. spegazzinii* parent that could conceal the expression of minor, quantitative loci.

RFLP analysis

The level of heterozygosity found with RFLP analysis in *S. spegazzinii* was lower than the level of heterozygosity found in *S. tuberosum*. Both species are self-incompatible so the difference could be due to the fact that *S. tuberosum* has been crossed with several wild species for improvement of the crop which resulted in a highly polymorphic species (Gebhardt et al. 1989), whereas the *S. spegazzinii* accession used here has apparently a much smaller gene pool and, therefore, a lower level of heterozygosity.

Distorted segregation ratios were detected for 24% of the markers that were segregating for *S. spegazzinii* alleles. Skewed segregation has been reported previously and seemed to be associated with distinct parts of the chromosomes (Bonierbale et al. 1988 and Gebhardt et al. 1991). In this experiment *S. spegazzinii* was used as a male parent. The segregation of alleles from *S. tuberosum*, which was used as a female parent, was skewed in only 18% of the markers used (data not shown). We assume that the higher distortion in *S. spegazzinii* could be caused by selective elimination of male gametes (Rick, 1969).

Statistical analysis

The small number of RFLP markers displaying polymorphism in the F1 population did not allow the use of interval mapping (Lander and Botstein, 1989), since no contiguous RFLP linkage map could be constructed. However, it is also possible to test

single RFLP markers for association with quantitative traits by means of analysis of variance (e.g., Keim et al. 1990). We applied this method to examine the 29 segregating RFLP markers and were able to detect three markers, TG63, Ssp75 and TG30, that were significantly associated with the resistance trait. A significance level of 5% for association of an RFLP marker with Ro1 resistance was obtained both after ANOVA and after Mann-Whitney U-test (non-parametric test). We realise that this may lead to a high overall error rate. Therefore, these significant associations must be seen as strong indications of the presence of quantitative resistance loci. The fact that both linked markers on chromosome *11* are significantly associated with nematode resistance, strengthens the point that a QTL is located on chromosome *11*. The three significant markers identified two QTLs for resistance to *G. rostochiensis* pathotype Ro1. We propose to name these loci on chromosome *10* and *11* as *Gro*1.2 and *Gro*1.3, respectively, analogous to the monogenic resistance locus *Gro*1 on chromosome *7* against *G. rostochiensis* pathotype Ro1 (Barone et al. 1990).

Only 22% of the genetic variation can be explained with the two loci we found. There are two possible explanations for this. Firstly, although the RFLPs are linked to loci involved in nematode resistance, the distance between marker and locus can still be large. Cross-overs between marker and resistance locus will reduce the association found with ANOVA. Secondly, there may be other loci involved in Ro1 resistance that could not be detected with the experimentation employed. A larger F1 population and more segregating markers are needed to detect these QTLs. It is possible that the RFLP marker Ssp27, which was on the verge of significance for association with Ro1 resistance, is linked to a third locus but the distance between marker and locus may be too large to detect significant association. Additional markers, segregating for *S. spegazzinii* alleles are needed to investigate if a third locus on chromosome *4* is present, but because of the lack of heterozygosity in *S. spegazzinii* such markers are hard to obtain. A backcross population, with a higher level of heterozygosity will be more informative to find additional resistance loci and markers closer to the *Gro*1.2 and *Gro*1.3 loci.

The potato genome is homoeologous to the tomato genome and the numbering of the chromosomes is similar (Bonierbale et al. 1988; Gebhardt et al. 1991). Interestingly, in tomato, resistance loci to *Fusarium oxysporum* (*I2*) and *Stemphylum sp.* have also been mapped on chromosome *11* (Sarfatti et al. 1989; Behare et al. 1991, respectively). The *Fusarium oxysporum I2* locus in tomato is mapped on the long arm of chromosome *11* (Segal et al. 1992). The *Gro*1.3 resistance locus has also been mapped on this arm.

Interestingly, the monogenic *Gro*1 resistance locus (Barone et al. 1990) from *S. spegazzinii* is closely located to yet another *Fusarium* resistance locus, *I*1, (Sarfatti et al.1991) on chromosome *7* (Leonards-Schippers et al. 1992). It is striking that specific resistances to different pathogens have been mapped to the same chromosome regions. Maybe the organisation of the resistance genes found on the specific chromosome regions in plants is similar to the human MHC complex (Dangl, 1992). Analogous to the MHC complex these chromosome regions could have hotspots for recombination and therefore allow the creation of new resistance genes with different specificity.

This article described the identification and localisation of quantitative resistance loci to *G. rostochiensis* pathotype Ro1 by means of RFLPs. It also shows that the identified loci explain 22% of the genetic variation and that they have an additive gene action. To explain all the genetic variation a closer linkage of RFLPs to the identified Ro1 resistance loci is needed. Putative additional loci will be detected by screening a backcross population of a resistant F1 plant and the susceptible *S. tuberosum* parent with RFLP markers.

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CHAPTER 4

Quantitatively-inherited resistance to *Globodera pallida* is dominated by one major locus in *Solanum spegazzinii*

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ABSTRACT

A high level of resistance to *Globodera pallida* pathotypes Pa2 and Pa3 exists in *Solanum spegazzinii*, a wild relative of potato (*S. tuberosum* ssp. *tuberosum*). Here we report the mapping of loci involved in quantitatively-inherited nematode resistance with the use of RFLPs. One major locus, *Gpa*, was mapped on chromosome *5* and two minor loci on chromosome *4* and *7* of *S. spegazzinii*. Additionally, the contribution of the susceptible parent to nematode resistance was determined. The *Gpa* locus was solely responsible for the high resistance level found in the segregating population. However, the RFLP marker closely linked to this resistance locus showed a distorted segregation, with a shortage of plants having the resistance linked allele. Our results indicate that a prediction of the genetic constitution of a quantitative trait based solely on phenotypic observations can lead to erroneous conclusions.

INTRODUCTION

The potato cyst-nematode *Globodera pallida* (Jones et al. 1970 and Stone 1972) can cause severe damage to the potato crop. Three pathotypes of *G. pallida*, Pa1 to Pa3, can be distinguished with a set of differentials which are derived from backcrosses of *Solanum tuberosum* with *S. multidissectum* and with *S. vernei* (Kort et al. 1977). Most cultivated potato varieties (*S. tuberosum* ssp. *tuberosum*) do not posses a good resistance to this nematode but resistance against *G. pallida* has been found in wild *Solanum* species. *S. multidissectum* shows resistance to pathotype Pa1 due to the presence of one major gene, *H2* (Dunnet 1961). A gene-for-gene relationship between this resistance gene and a *G. pallida* avirulence gene has been described by Parrott (1981). Resistance to pathotypes Pa2 and Pa3 was found in *S. tuberosum* ssp *andigena* CPC 2802. This resistance was first thought to be monogenic (Howard et al. 1970), but was later proven to be polygenic (Dale and Phillips 1982). Another source of resistance to *G. pallida* is *S. vernei*, which has a quantitative, presumably polygenic, resistance (Ross 1986).

Recently, Arntzen et al. (1993) described a qualitative and monogenic resistance to Pa2 population D236. This resistance was derived from *S. tuberosum* ssp *andigena* CPC1673. So far, this locus only gives resistance to one population of pathotype Pa2, namely D236, and not to other Pa2 or Pa3 populations tested (Arntzen et al. 1992).

New sources with broad spectrum resistance to *G. pallida* as well as *G. rostochiensis* have been searched for in other wild *Solanum* species (Van Soest et al. 1983; Dellaert and Hoekstra 1987; Jackson et al. 1988; Turner 1989). Most resistances of these wild species showed a quantitative inheritance, suggesting the action of several genes (Dellaert et al. 1988). *S. spegazzinii* 8218-15 was one of the resistant accessions that had been studied in detail. Dellaert et al. (1988) assumed that the resistance to *G. pallida* pathotype Pa3 was dominated by 2 to 3 major genes, each with incomplete resistance and in addition they predicted the presence of minor genes.

Quantitative traits can be dissected into discrete genetic factors with the use of detailed RFLP linkage maps; all regions of the genome can be assayed and accurate estimates of phenotypic effects and genetic positions can be derived (Paterson et al. 1988). In this paper we describe the identification and mapping of loci, involved in resistance to *G. pallida* pathotypes Pa2 and Pa3, derived from *S. spegazzinii* 8218-15. The aim of this study was to examine the assumptions about the genetic basis of resistance made by Dellaert et al. (1988). In addition we investigated the possibility of the presence of QTLs, contributing to the nematode resistance, from the susceptible parent *S. tuberosum*.

MATERIALS AND METHODS

Plant material

S. spegazzinii BGRC accession 8218 seedling number 15 (Sspeg)was used as pollinator in a cross with the diploid susceptible *S. tuberosum* SH 78-88-1320 (Stub). The F1 progeny were tested for resistance to *G. pallida* pathotypes Pa2 and Pa3 (see below). The *S. spegazzinii* parent appeared to be heterozygous at the resistance loci because resistance to pathotypes Pa2 and Pa3 was segregating in the F1 progeny. Mapping of the loci involved in resistance with RFLPs was, therefore, performed in this F1 population.

RFLP analysis

The RFLP markers that were used in this study and the RFLP analysis have both been described earlier by Kreike et al. (1993).

Resistance test

The resistance test for pathotype Pa2 was done in 1988 on 96 F1 plants (total F1 progeny) with nematode population P2-22 (Arntzen and Van Eeuwijk 1992) and for pathotype Pa3 in 1987 (44 plants) and 1989 (39 plants) with nematode population Coll. 1077 (Arntzen and Van Eeuwijk 1992). The F1 population was also tested for resistance against *G. rostochiensis* pathotype Ro1, which is described by Kreike et al. (1993). The resistance tests were performed in five replications in a randomized block design. Thirty cysts enclosed in a nylon net were used as inoculum. After 5 months the newly formed cysts were collected and counted. A more detailed description of the resistance test is given by Kreike et al. (1993). The differentials VT(N)^2 62-33-3 and AM78-3778 were used to discriminate between the *G. pallida* pathotypes Pa2 and Pa3 used in this study.

Statistical analysis

A normalizing transformation [^{10}log(x+1)] was performed on the counts of the newly-formed cysts per replicate. These transformed data of the resistance test were investigated with analysis of variance (ANOVA) for a randomized block design. From this ANOVA the heritability of the genotypic mean values for each pathotype was calculated as described by Kreike et al. (1993). The association of marker genotypes with resistance was assessed with one-way ANOVAs based on the averages over the five replicates of the log-transformed cyst counts. In these ANOVAs the data were classified with the alleles of each parent seperately i.e., for each markers having two *S. spegazzinii* and two *S. tuberosum* alleles two seperate ANOVAs were performed. A significance level of $P < 0.05$ was employed. The magnitude of the marker associated phenotypic effect is described by the coefficient of determination (R^2), which represents the fraction of the total variance accounted for by the marker genotypes. Since the ANOVA assumes a normal distribution of the data within the classes, and this assumption might be violated, the ANOVA results were verified with a nonparametric test (Mann-Witney U-test).

An RFLP linkage map of chromosome 5 was constructed with the computer program JoinMap (Stam 1993). Interval mapping, as described by Lander and Botstein (1989), could be performed for chromosome 5 for the alleles from the *S. spegazzinii*

parent, by treating the F1 as a first generation backcross progeny with fully homozygous genotypes. This analysis was done using the computer program MapQTL, written by Van Ooijen. Readers who are interested in receiving MapQTL should contact Van Ooijen fur further details (E-mail [internet]: j.w.van.ooijen@cpro.agro.nl).

RESULTS AND DISCUSSION

Resistance test

The genetic variance in the F1 for both Pa2 and Pa3 resistance was very significant (for both P <0.0001), which means that the different levels of Pa2 and Pa3 resistance found in the F1 population had a genetic basis. The heritability of the genotypic mean values for resistance to *G. pallida* was very high, 0.88 for Pa2 and 0.89 for Pa3 (both 1987 and 1989 experiments). A high correlation (r=0.65) was detected between Pa2 and Pa3 resistance. This indicated either genes with pleiotropic effects, or closely linked genes. No significant correlations (Pa2-Ro1: r=0.17 and Pa3-Ro1: r=0.10) were detected between resistances to *G. pallida* and *G. rostochiensis* (h^2 =0.63) (Kreike et al., 1993) and therefore different, unlinked loci are assumed to be involved in resistance for the two nematode species.

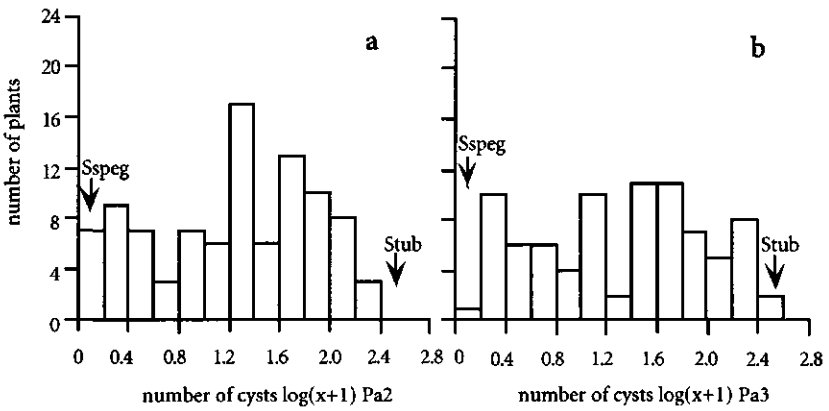

Figure 1. Frequency distribution of the mean number of cysts found on the F1 plants with Pa2 (a) and Pa3 (b) resistance tests. The data of the resistance tests were transformed [^{10}log(x+1)].

Figure 1 shows the frequency distribution of Pa2 and Pa3 resistance in the F1 population. A continuous range of cyst numbers was found in this population for both pathotypes. Hence, no qualitative inheritance can be detected. The average newly-formed number of cysts for the parents, *S. spegazzinii* and *S. tuberosum*, were 1 and 300, respectively. The differential VT(N)^2 62-33-3 was susceptable for Pa3 (on average 128 cysts) while AM78-3778 was resistant for Pa3 (on average 1,2 cysts) but susceptable for Pa2 (on average 63 cysts)

RFLP analysis

One undred and seven RFLP markers were tested for heterozygosity within the parental genotypes using four restriction enzymes. This search yielded 29 RFLP markers heterozygous for *S. spegazzinii* and 43 RFLP markers for *S. tuberosum*. Table 1 shows the number of heterozygous markers per chromosome for each parent. The segregation of these markers was determined in the F1 population and used for linkage analysis, which is described by Kreike et al. (1993).

Table 1. Number of polymorphic markers used per chromosome to detect association with nematode resistance from *S. spegazzinii* and *S. tuberosum*.

Chromosome	*S. spegazzinii*	*S. tuberosum*
1	1	8
2	1	5
3	2	2
4	2	1
5	5	4
6	2	0
7	4	3
8	0	3
9	2	5
10	2	3
11	2	2
12	5	4
not mapped	1	4
total	29	43

Mapping of the resistance loci

The association of marker genotypes with nematode resistance was assessed with one-way ANOVAs, of which the results are shown in Table 2. Significant associations ($P<0.05$) indicated that the RFLP marker was linked to a resistance locus. As a

verification, associations were also determined with a nonparametric test and these confirmed the results found with the one-way ANOVAs.

The RFLP markers, Ssp124, Ssp112, Ssp37 and Ssp72, with the highest significance for association with nematode resistance were all located on chromosome 5 (Table 2) and showed association with *G. pallida* pathotype Pa2, as well as pathotype Pa3, resistance. Also two markers on chromosome 7, TG143 and Ssp51, showed association with resistance to both pathotypes. On chromosome 4, however, marker Ssp27 was associated only with resistance to *G. pallida* pathotype Pa3.

Table 2. Association between RFLP markers and nematode resistance after ANOVA and explained variance per RFLP marker in % of the total phenotypic variance of clone means. P-values that are given in parentesis are considered not significant. Abbreviations: Chr= chromosome; Segr= segregation; Expl. var.= explained variance.

Marker	Chr	Segr Ratio	P value Pa2	P value Pa3	Expl var Pa2	Expl var Pa3
S. spegazzinii						
Ssp27	*4*	47:40	(0.50)	0.03	-	5
Ssp124	*5*	29:22	0.005	0.02	14	9
Ssp112	*5*	37:30	0.0008	0.002	15	13
Ssp37	*5*	49:41	0.0001	0.0001	27	22
Ssp72	*5*	56:24#	0.0001	0.0001	51	45
TG142	*7*	48:33	0.03	0.02	5	7
Ssp51	*7*	37:33	0.03	0.007	6	9
S. tuberosum						
Ssp56	*1*	10:32#	0.03	(0.11)	9	-
TG130	*3*	21:33	(0.06)	0.03	-	7
Ssp37	*5*	34:42	(0.09)	0.03	-	5
Ssp75	*11*	17:19	(0.22)	0.05	-	9
TG68	*12*	17:20	0.03	(0.27)	10	-
total*					76	80

distorted segregation

* The highest explained variances for each chromosome are added up to give the total explained variance

In order to obtain an estimate of the map position of the resistance locus, interval mapping (Lander and Botstein 1989) was performed for chromosome 5 in which an RFLP map could be constructed. The F1 population was treated as a first generation backcross population by using the two *S. spegazzini* alleles for each marker only. In this way only the segregation of resistance loci heterozygous in *S. spegazzinii* is studied. The results from the interval mapping on chromosome 5 are presented in Fig. 2a. The

threshold was set at LOD 3.0 according to Van Ooijen (1992). These results show that there is most probably one major resistance locus active against both Pa2 and Pa3, which is located in between the markers Ssp37 and Ssp72, approximately 10 cM from Ssp72. We propose to name the major resistance locus on chromosome *5*, *Gpa*, since it is involved in resistance to *G. pallida* pathotype Pa2 as well as Pa3 (Fig. 2b).

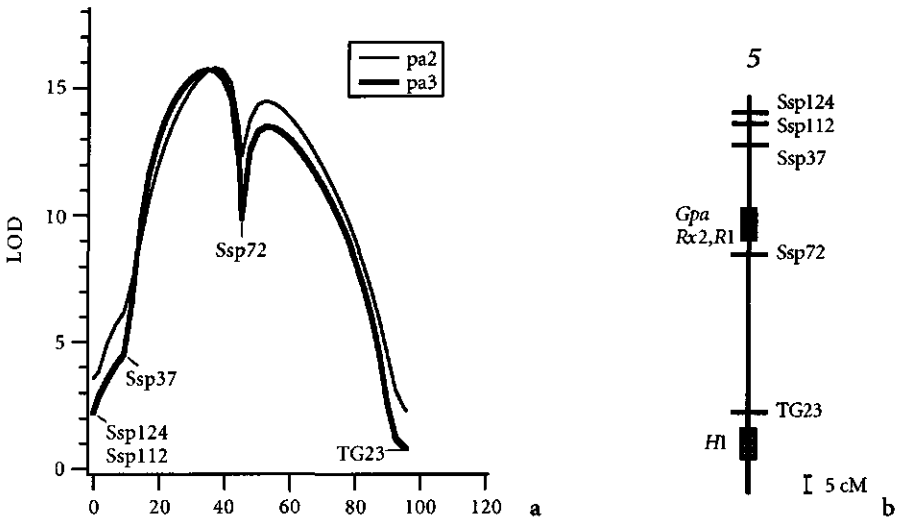

Figure 2a. LOD scores for *G. pallida* pathotype Pa2 ad Pa3 resistance on chromosome 5 of *S. spegazzinii*.. 2b. Position of the Gpa locus on chromosome 5 and the location of other resistance loci on this chromosome (Ritter et al. 1991; Leonards-Schippers et al. 1992; Gebhardt et al. 1993; Pineda et al. 1993).

The existence of loci, heterozygous for alleles involved in nematode resistance, in the susceptible parent *S. tuberosum*, was also determined. Indications for the presence of QTLs were found on chromosome *1, 3, 5, 11* and *12* (Table 2). Since the segregation of the corresponding RFLP alleles was determined on a limited number of plants only, these associations have to be seen as mere indications for the presence of loci contributing to nematode resistance. The existence of these loci, in the susceptible *S. tuberosum* ssp *tuberosum* has been described previously. Dale & Phillips (1985) found variation in nematode resistance level within *S. tuberosum* ssp *tuberosum* genotypes, ranging from fully susceptible to fully resistant.

It is possible that the *Gpa* locus does not consists of one locus that is responsible for resistance against both pathotypes but consists of two closely-linked loci, each conferring resistance to an individual pathotype. Four F1plants were found which were resistant ($^{10}\log(x+1) < 1.0$) to one pathotype only and susceptable ($^{10}\log(x+1) > 1.0$) to the other (Table 3). The presence of the *Gpa* locus can be determined with the RFLP markers Ssp72 and Ssp37, which are surrounding the resistance locus. For two plants the presence of this locus can not be determined because the RFLP pattern of Ssp72, which is closely linked to the locus, is not known. Based on the RFLP markers, plant SP1 did not possess the *Gpa* locus, therefore the high resistance level to pathotype Pa2 could be caused by the action of several minor loci. Based on marker Ssp72, plant V223 most likely possessed the *Gpa* locus, but has a recombination between marker Ssp72 and Ssp37, for which it has the susceptibility-linked allele. The recombination event could have taken place at the *Gpa* locus, separating the Pa2 and the Pa3 pathotype specific resistance loci.

Table 3. Mean cyst numbers of F1 plants which are resistant to only one pathotype and susceptible to the other, and RFLP data for markers Ssp72 and Ssp37 on chromosome 5. In brackets the mean [$^{10}\log(x+1)$] transformed cyst numbers are given with the standard error. 1= presence of the resistance linked RFLP allele; 0= absence; nd= not determined.

F1- Genotype	cysts Pa2	cysts Pa3	Ssp72	Ssp37
SP17	101.4	9.0	nd	0
	(2.01+0.19)	(1.00+0.19)		
V28	100.2	3.5	nd	1
	(2.01+0.21)	(0.65+0.21)		
SP1	5.6	85.3	0	0
	(0.82+0.19)	(1.94+0.19)		
V223	4.9	42.9	1	0
	(0.77+0.21)	(1.61+0.21)		

A large part of the total phenotypic variance could be explained with the associated RFLP markers (Table 2). Marker Ssp72, which is linked to a major locus, explains 51 % and 45 % of the total variance of Pa2 and Pa3 resistance, respectively. The other markers on chromosome *5* all showed linkage with the same major resistance locus. The markers on chromosomes *4* and *7* from *S. spegazzinii* and on chromosome *1*, *3*, *5*, *11* and *12* from *S. tuberosum* explained 10% or less of the total variance and are therefore considered to be minor loci. Because we were not able to employ more segregating markers, we could not investigate whether the low explained variance of some markers was due to either a large distance between marker and resistance locus, or to a small genotypic effect of a closely-linked locus. On the other hand, adding up the explained

variance of all significant and unlinked markers leads to a total of 76% and 80% of the explained variance for Pa2 and Pa3 respectively (Table 2). And because the heritability for Pa2 and Pa3 resistance was 0.88 and 0.90, respectively, it seems that most of the genetic variantion can be explained with these markers. Hence, we do not expect much larger genotypic effects of the loci linked to these markers.

Classical segregation analysis versus genetic analysis with markers

Based on the research of Dellaert et al. (1988) we expected to find two to three major loci which are involved in resistance to *G. pallida*. However only one major locus was detected. There are several reasons for this discrepancy. First, Dellaert et al. performed segregation analysis on 44 plants only which is the population tested against Pa3 in 1987 also used in this paper (see M&M section). A population size of only 44 plants is relatively small for segregation analysis on a quantitative trait such as the present nematode resistance. Secondly, segregation analysis of a continuously-distributed trait relies heavily on the choise of the threshold for genotype classification (e.g., resistant or susceptible). Genetic analysis based on (molecular) markers circumvents the choise of genotype classification. Thirdly, classical segregation analysis assumes segregation to be according to the Mendalian ratios. The estimation of the number of genes is based on the best fitting segregation ratio, given the chosen-genotype classification and genetic model. Minor deviations are often explained by adjusting the genetic model, for instance, by assuming incomplete dominance or linkage. When, however, genetic analysis with markers is performed, the realised segregation can be determined, and possible distortion can even be tested.

We found that the *Gpa* locus is situated in a region where segregation is distorted. Marker Ssp72, which is closely linked to the resistance locus, showed a skewed segregation with a shortage of the resistance-linked allele (Table 2, Fig. 3). The choise of the genotype classification threshold based on Fig. 1 is quite arbitrary; thus, our results demonstrate that classical segregation analysis can easily lead to erroneous conclusions.

Distorted segregation in this region of potato chromosome 5 has been observed previously (Ritter et al. 1991). Resistance to PVX also showed a segregation ratio that deviated significantly from 1:1 in the F1 progeny. Additionally in this case a marker was found on chromosome 5 which cosegregated with the resistance trait. Several mechanisms that cause distorted segregation are known, such as incompatibility, male

/female gamete selection or even sporophytic selection. The precise mechanism causing this disturbance at chromosome 5 remains to be elucidated.

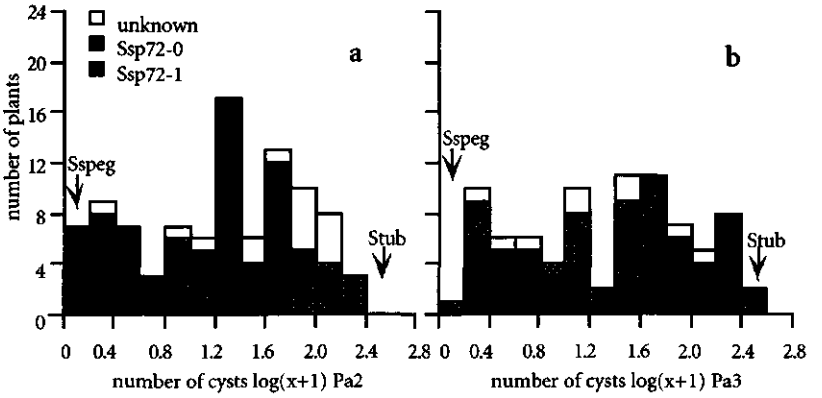

Figure 3. Presence of the alleles of marker Ssp72, which is closely linked to the *Gpa* locus, in the frequency distribution of Pa2 (a) and Pa3 (b) resistance in the F1 population.

Other resistance loci on chromosome 5

The *Gpa* locus on chromosome 5 is yet another nematode resistance locus on this chromosome (Fig 2b). The *H*1 gene from *S. tuberosum* ssp *andigena* CPC 1673 which conferred resistance to *G. rostochiensis* pathotype Ro1 was also mapped on chromosome 5, near TG23 (Gebhardt et al. 1993; Pineda et al. 1993). Furthermore the locus determining resistance to the D236 population of *G. pallida* pathotype Pa2 is probably located on chromosome 5 too, because Arntzen et al. (1993) assumed linkage with the *H*1 locus. Resistance loci against other pathogens of potato have also been mapped on this chromosome, e.g., the *Rx*2 locus against PVX (Ritter et al. 1991), the *R*1 locus against *Phytophthora infestans* (Leonards-Schippers et al. 1992) and trichome-mediated insect resistance (Bonierbale et al. 1993)(Fig 2b). The location of the latter could not be shown in Figure 2b, because other markers were used to map this resistance, but its location is most likely in the same region as the *Gpa* locus.

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CHAPTER 5

Mapping of QTLs involved in nematode resistance, tuber yield and root development

CM Kreike, AA Kok-Westeneng, JH Vinke, WJ Stiekema

ABSTRACT

A backcross population, derived from the cross (*S. tuberosum* x *S. spegazzinii*) x *S. tuberosum* was used to map QTLs involved in nematode resistance, tuber yield and root development. Complete linkage maps were available for the interspecific hybrid parent as well as the *S. tuberosum* parent and interval mapping for all traits was performed for both. Additionally the intra- and interlocus interactions of the QTLs were examined. The *Gro*1.2 locus, involved in resistance to *G. rostochiensis* pathotype Ro1 that was previously mapped in the *S. tuberosum* x *S. spegazzinii* F1 population, could be located more precisely on chromosome *10*. A new resistance locus *Gro*1.4, also conferring resistance to *G. rostochiensis* pathotype Ro1, was found on chromosome *3*. Different alleles of this locus originating from both parents contributed to the resistant phenotype, indicating multiallelism at this locus. No epistasis was observed between these two resistance loci. For resistance to *G. pallida* no QTLs were detected. QTL mapping for factors involved in tuber yield yielded only one minor QTL on chromosome 4. Two QTLs with large effects were mapped on chromosome *2* and *6* involved in root development. An epistatic interaction was found between these two loci.

INTRODUCTION

Quantitative traits are characterised by a continuous distribution of the phenotypic value. This continuous variation is generally assumed to be due to the effects of multiple genes, each of them indicated as a quantitative trait locus (QTL), and environmental effects (Johanssen 1909; Nilsson-Ehle 1919; East 1915). The early attempts to link a QTL to a qualitative gene, in this case a morphological marker, was described by Sax (1923) and in greater detail by Thoday (1961). However, for most organisms only a few neutral qualitative monogenic markers are available and only after the development of molecular markers and the construction of molecular marker linkage maps (Botstein et al. 1980), more extensive attempts have been made to identify and localise QTLs.

If no complete linkage map is available, linkage between the markers and the QTLs can be detected with single point analysis (Keim et al. 1990; Kreike et al. 1993 and 1994). However, the availability of a complete linkage map allows the use of the more powerful interval mapping (Lander and Botstein 1989). Several quantitative traits like soluble solids content, fruit pH and -weight and seed weight in tomato and resistance to *Phytophthora infestans* in potato, have been studied with this method (Paterson et al. 1988 and 1991; De Vicente and Tanksley 1993; Leonards-Schippers et al. 1994).

QTLs for cyst nematode resistance in potato have been mapped previously by Kreike et al. (1993 and 1994). The QTLs were segregating in an F1 population of a cross between a di(ha)ploid *Solanum tuberosum* with the wild potato species *S. spegazzinii.* Two QTLs, *Gro*1.2 and *Gro*1.3, involved in resistance to *Globodera rostochiensis* pathotype Ro1 were found on chromosomes *10* and *11* (Kreike et al. 1993) and a major QTL, *Gpa*, involved in resistance to *G. pallida* pathotype Pa2 and Pa3 was found on chromosome *5* (Kreike et al. 1994).

In this paper we describe the mapping of nematode resistance loci in a backcross (BC) population derived from F1 genotype F1-38, obtained from the above mentioned *S. tuberosum* x *S. spegazzinii* cross, and *S. tuberosum.* From RFLP analyses of the F1 population, it was known that genotype F1-38 was most likely heterozygous at the *Gro*1.2 and *Gro*1.3 loci but homozygous susceptible at the major *Gpa* locus. The BC population, therefore, would enable us to confirm and fine-map the *G. rostochiensis* QTLs and locate minor QTLs involved in *G. pallida* resistance. This BC population enabled us to map also QTLs from the *S. spegazzinii* parent involved in nematode resistance that were not segregating in the F1 population. In addition to nematode resistance, QTLs for tuber yield and root development were mapped.

Another objective of this research was to determine the intra- en interlocus interactions of the mapped QTLs. In a mapping analysis of inbreeding species, the segregation of only two alleles can be followed, whereas in a heterozygous species like di(ha)ploid *Solanum,* up to 4 different alleles, 2 from each parent, may be distinguished at a locus. Intralocus interaction has not been studied in great detail with the exception of Van Eck et al. (1994). Stuber et al. (1992) described overdominant gene action at single QTLs in maize. In our BC population up to three different alleles could segregate at a QTL and the interaction between these alleles was studied.

Interaction between the QTLs (interlocus interaction) was studied by Fatokun et al. (1992). He described epistasis between some of the QTLs, but mostly the QTLs displayed

no interaction. In this paper the interactions of the QTLs involved in nematode resistance, tuber yield and root development are also examined.

MATERIALS AND METHODS

Plant material

Clone F1-38 is an interspecific hybrid between a di(ha)ploid *S. tuberosum* SH 78-88-1320 (Stub) and *S. spegazzinii* BGRC 8218-15 (Sspeg). F1-38 was used as a female parent in a backcross to Stub. The BC progeny of 80 plants was tested for resistance to *G. rostochiensis* pathotype Ro1 and *G. pallida* pathotypes Pa2 and Pa3 (see below).

RFLP analysis

The RFLP markers that were used in this study and the RFLP analysis have been described earlier by Kreike et al. (1993). For the interspecific hybrid parent 68 markers were used and for the Stub parent 38 markers.

Analysis of the quantitative traits

The resistance tests with 80 BC plants was carried out in 1991 for *G. rostochiensis* pathotype Ro1 and *G. pallida* pathotypes Pa2 and Pa3 with nematode populations M'BA, P2-22 and Coll. 1077, respectively. (Arntzen and Van Eeuwijk 1992). Each of the three resistance tests were performed in 5 replications in a randomized block design. Thirty cysts enclosed in a nylon net were used as inoculum. After 5 months the newly formed cysts were collected and counted and the total cyst weight per plant was determined. A more detailed description of the resistance test is given by Kreike et al. (1993).

A normalizing transformation [$^{10}\log(x+1)$] was performed on the number of cysts (= NC) and the total cyst weight (= TCW) data to investigate these data with analysis of variance (ANOVA). From these ANOVAs the heritabilities of NC and TCW under the different nematode infections was calculated as described by Kreike et al. (1993). For QTL mapping the data from the 5 replications were averaged. The frequency distribution is shown in Figure 1.

After the resistance test the number of tubers, the total tuber weight and the development of the root system were determined. The latter was scored on an ordinal scale from 1 (poor root system) to 3 (good root system).

To obtain normality for ANOVA, a square root transformation was performed on the number of tubers (= NT) and total tuber weight (= TTW) data. No transformation was necessary for root development (RD). The three nematode infections had no significant effect on the NT, TTW and RD after ANOVA and therefore the heritability was determined using the 15 replications. For QTL mapping of NT, TTW and RD, the data of the 15 replications were averaged and their frequency distribution is shown in Figure 1.

Correlations were calculated between the quantitative traits to detect any relationship between the various characters. The calculations were performed with the quantitative data that were used for QTL mapping.

QTL mapping

Due to heterozygosity in both parents, RFLP linkage maps could be constructed of F1-38 as well as Stub (Kreike et al. 1995). For most markers the segregation of the alleles per parent could be considered as a first generation BC population with fully homozygous parents, which allowed the application of interval mapping, as described by Lander and Botstein (1989), but also F2-type segregating markers were found. The parental linkage maps were made with JoinMap (Stam 1993), using BC- and F2-type segregating markers. QTL mapping on the linkage maps of both parents was done using the computer program MapQTL (JW Van Ooijen, pers comm.). This program has two options for mapping, single point analysis using the nonparametric Kruskal-Wallis test and interval mapping. First, the Kruskal-Wallis test was used to search the chromosomes for markers significantly associated with the quantitative traits ($P< 0.01$). This was done for both types of segregating markers. Then interval mapping was performed on only the BC-type segregating markers. A LOD value over 2 (Van Ooijen 1992) and a contribution to the total phenotypic variance (R^2) of >15% were taken as an indication that a segregating QTL was present.

Analysis of intra- and interlocus interaction

For intralocus interactions three different alleles can be monitored in the segregation analysis; a *S. spegazzinii* allele from the hybrid F1-38 (speg-allele), a *S. tuberosum* allele derived from the hybrid and the *S. tuberosum* parent (tub1-allele), which is identical by descent, and a unique *S. tuberosum* allele derived from *S.*

tuberosum (tub2-allele). In case of linkage we presume that the tub1-allele is linked to the same QTL allele.

For the determination of the intra- and interlocus interactions, the markers nearest to the QTL were taken to perform two- or three- way ANOVAs. From the two-way tables, which present the phenotypic values of the resulting marker genotype classes, the contribution of an allele to a trait could be deduced. A significant interaction term after ANOVA was taken to indicate epistasis.

RESULTS

Analysis of the quantitative traits

The frequency distribution for the measured traits, NC, TCW, NT, TTW and RD in the BC population and the values of Sspeg, Stub and F1-38 are given in Figure 1.

All traits show a continuous distribution indicating their quantitative nature. For some traits transgressive phenotypes were observed in the BC population. Correlation between the different quantitative traits was calculated to observe any relationships between the various characters studied (Table 1). As is to be expected the traits NC and TCW were highly correlated ($r > 0.97$). Also NT was highly correlated with TTW ($r = 0.91$). Subsequently only NC and NT will be described in this paper. The heritabilities for the quantitative traits are given in Table 2a and b and were very high for NT and RD.

Table 1. Correlation between the quantitative traits, NC and TCW under the different nematode infections, NT, TTW and RD.

	NC Ro1	NC Pa2	NC Pa3	TCW Ro1	TCW Pa2	TCW Pa3	NT	TTW
NC Ro1								
NC Pa2	.24							
NC Pa3	.28	.59						
TCW Ro1	.97	.31	.28					
TCW Pa2	.25	.99	.56	.34				
TCW Pa3	.31	.59	.99	.32	.58			
NT	.25	.27	.39	.25	.29	.40		
TTW	.31	.28	.45	.31	.31	.47	.91	
RD	.47	.36	.43	.46	.37	.48	.42	.51

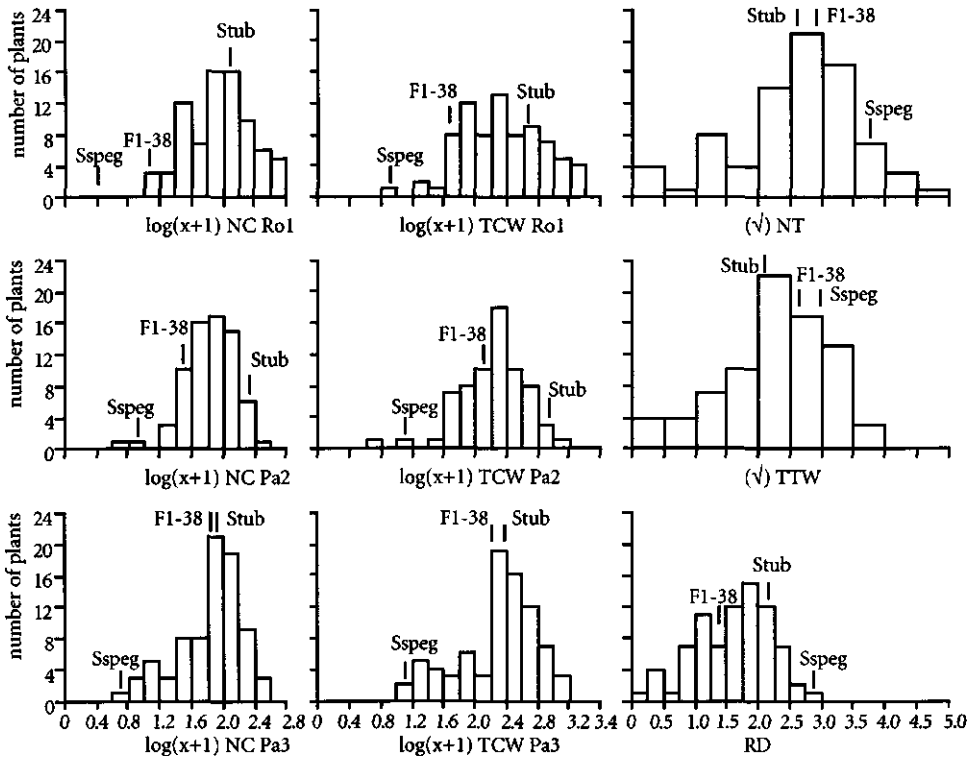


Figure 1. Frequency distribution of the quantitative traits, NC [^{10}log(x+1) number of cysts], TCW [^{10}log(x+1) total cyst weight] under the different nematode infections, and NT (square root transformed number of tubers), TTW (square root transformed total tuber weight) and RD (root development) in the BC population. The phenotypic values of *S. spegazzinii* (Sspeg), *S. tuberosum* (Stub) and F1-38 are indicated.

RFLP map

Little differences in the map positions of several markers as described by Kreike et al. (1995) can be found on chromosomes *3*, *4*, *5* and *10* of parent F1-38. The differences on chromosome *3* (TG134-TG42), *4* (Ssp61-Ssp118b- Ssp47) and *5* (Ssp72-Ssp118a-GP21a) can be attributed to the low recombination frequency between these markers in the F1-38 parent. A different order of these markers was found in other parents with higher recombination frequencies that enabled a more accurate mapping (Kreike et al.

1995). The ambiguous position of marker Ssp106 on chromosome *10* is a result of its F2-type segregation and the inaccuracy to determine the recombination frequency between F2-type and BC-type segregating markers.

Table 2a. Heritability of the quantitative traits and the map location of the QTLs with their nearest marker, segregating from parent F1-38. The LOD score and the R^2 at the QTL position are given. Abbreviations: h^2= heritability; Chr= chromosome; R^2= variance explained by QTL; Marker= marker nearest to QTL

Trait	h^2	Chr	QTL position (cM)	LOD score	R^2	Marker	Marker position (cM)
NC Ro1	0.76	*3*	22.5	3	24	Ssp8	35
		10	51#	P=0.005	19.5	Ac15-7	51
NC Pa2	0.58						
NC Pa3	0.64						
NT	0.92	*4*	45	2.3	16	Ssp47	40
RD	0.91	*2*	30	2.6	32	TG34	47.5
		6	25	2.3	41	TG118	0

The position of this QTL is not determined with interval mapping but with Kruskal-Wallis test and therefore the P -value is given

Table 2b. Heritability of the quantitative traits and the map location of the QTLs with their nearest marker, segregating from parent Stub. The LOD score and the R^2 at the QTL position are given. Abbreviations: h^2= heritability; Chr= chromosome; R^2= variance explained by QTL; Marker= marker nearest to QTL

Trait	h^2	Chr	QTL position (cM)	LOD score	R^2	Marker	Marker position (cM)
NC Ro1	0.76	*3*	25	2.1	20	TG130	37.5
NC Pa2	0.58						
NC Pa3	0.64						
NT	0.92						
RD	0.91						

QTL mapping

The computer program MapQTL (JW Van Ooijen, pers comm.) was used for mapping the quantitative traits. A Kruskal-Wallis test was performed on all markers individually. If significant associations (P<0.01) were found between a marker and a trait, interval mapping of the chromosomes containing these markers was performed. Interval mapping could only be performed for the BC-type segregating markers. The results of the interval mapping are given in Table 2a+b and Figure 2.

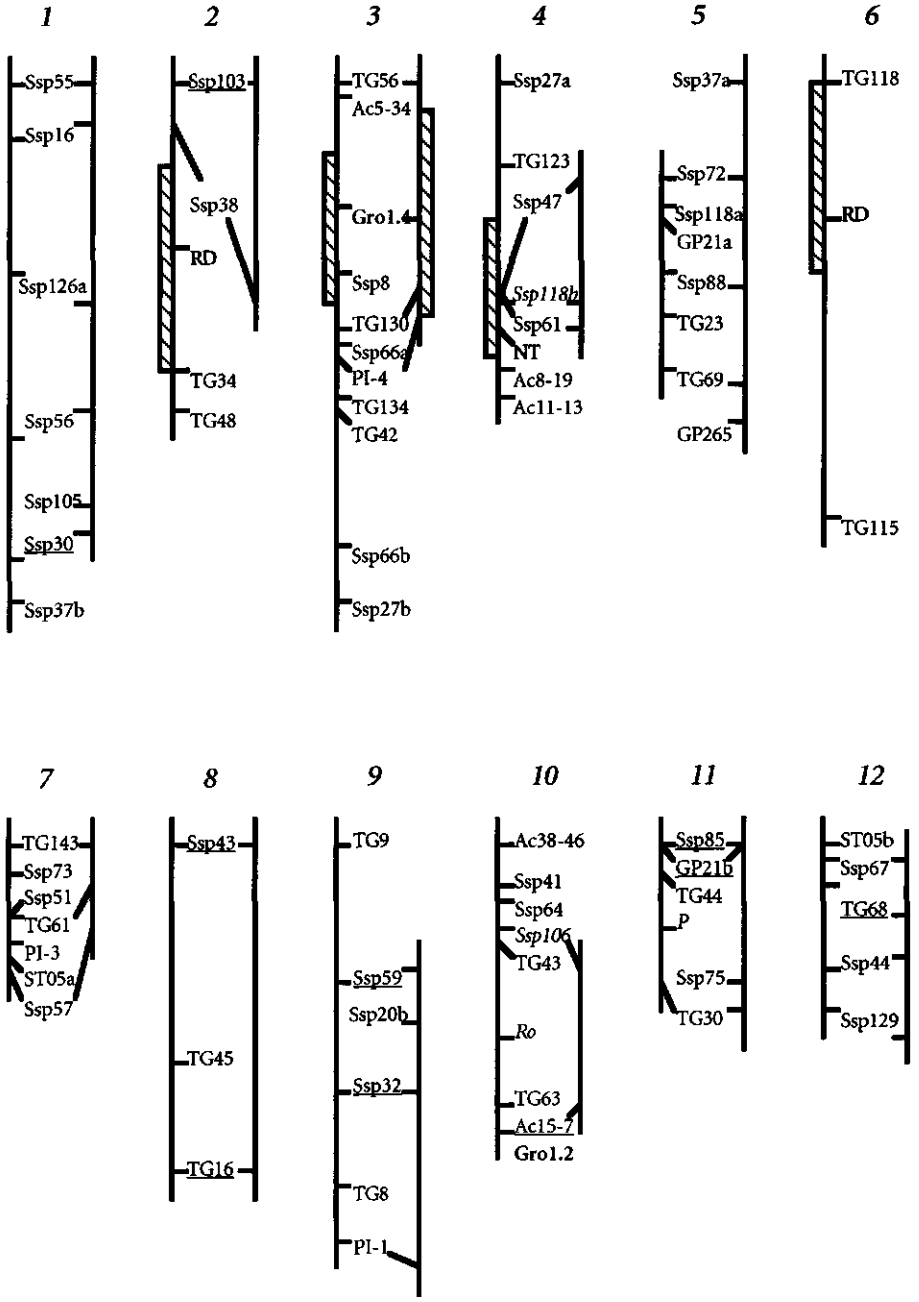
For resistance against *G. rostochiensis* pathotype Ro1, determined as NC-Ro1, two QTLs were found, one on chromosome *3* and one on chromosome *10*. The presence of a QTL on chromosome *10*, *Gro*1.2, segregating from *S. spegazzinii* BGRC 8218-15, conferring resistance to pathotype Ro1, was described earlier by Kreike et al. (1993). The position of this QTL, in the proximity of TG63, is more distal, and closer to marker Ac15-7 (Figure 2). The exact location of this QTL could however not be determined with interval mapping since marker Ac15-7 had an F2-type segregating pattern. The contribution of this locus to the total phenotypic variance was 19.5%. The other QTL involved in Ro1 resistance was observed on chromosome *3* and alleles from both parents contributed to the trait (Figure 2). The phenotypic variance that could be explained by this locus was 24% for F1-38 alleles and 20% for Stub alleles. We propose to name this locus, the *Gro*1.4 locus, in correspondence with the previously reported resistance loci to *G. rostochiensisis* pathotype Ro1, derived from *S. spegazzinii* (Barone et al. 1990; Kreike et al. 1993). No QTL was found on chromosome *11*, in the region of the *Gro*1.3 locus. This locus was mapped in the F1 population (Stub x Sspeg), and described by Kreike et al. (1993).

QTL mapping of NC-Pa2 and NC-Pa3 revealed no QTLs. Only on chromosome *5*, a possible QTL involved in resistance to *G. pallida* pathotype Pa3, could be located near marker Ssp88, segregating from the Stub parent. The LOD score was just below the threshold level (LOD=1.8, R^2=15%).

For NT, QTL analysis discovered only one QTL that was located on chromosome *4*, near marker Ssp47, derived from F1-38, with an effect of 16%.

Analysis for RD yielded two QTLs from F1-38 with large effects. One locus on chromosome *2*, near marker TG34, which explained 32% of the total phenotypic variation and another locus on chromosome *6*, near marker TG118, which explained 41%.

Figure 2. RFLP maps of the parents F1-38 (left bar) and Stub (right bar). Underlined markers had an F2-type segregation patterns and were not used for interval mapping. Markers in ithalic had an F2-tyoe segregation for F1-38 alleles and a BC type segregation for pattern for Stub alleles and were only used in parent Stub for interval mapping. The QTLs are shown in bold an the hatched box indicated the 1 LOD support interval.


1
Ssp55
Ssp16
Ssp126a
Ssp56
Ssp105
Ssp30
Ssp37b
2
Ssp103
Ssp38
RD
TG34
TG48
3
TG56
Ac5-34
Gro1.4
Ssp8
TG130
Ssp66a
PI-4
TG134
TG42
Ssp66b
Ssp27b
4
Ssp27a
TG123
Ssp47
Ssp118b
Ssp61
NT
Ac8-19
Ac11-13
5
Ssp37a
Ssp72
Ssp118a
GP21a
Ssp88
TG23
TG69
GP265
6
TG118
RD
TG115
7
TG143
Ssp73
Ssp51
TG61
PI-3
ST05a
Ssp57
8
Ssp43
TG45
TG16
9
TG9
Ssp59
Ssp20b
Ssp32
TG8
PI-1
10
Ac38-46
Ssp41
Ssp64
Ssp106
TG43
Ro
TG63
Ac15-7
Gro1.2
11
Ssp85
GP21b
TG44
P
Ssp75
TG30
12
ST05b
Ssp67
TG68
Ssp44
Ssp129

Analysis of intra- and interlocus interaction

Intralocus interactions at a QTL could be examined if both parents were heterozygous at the associated marker locus. For resistance to *G. rostochiensis* pathotype Ro1, QTLs on chromomose *3* and *10* were found, respectively the *Gro*1.4 and *Gro*1.2 loci. The QTL on chromosome *3* is linked closest to marker Ssp8, segregating from F1-38 and TG130 segregating from Stub (Figure 2, Table 2a+b). The segregation data of both markers were used to construct a two-way table (Table 3). Two-way ANOVA did not show a significant interaction, therefore the effect of the speg-allele from F1-38 and the tub1-allele from Stub was additive.

Table 3. Intralocus interaction at the *Gro*1.4 locus on chromosome 3 involved in resistance to *G. rostochiensis* pathotype Ro1. The two-way table is constructed with marker Ssp8 segregating from parent F1-38 and TG130 segregating from parent Stub. The ^{10}log(x+1) transformed NC observed in each marker-genotype class is given together with the number of plants (in parenthesis). The speg-allele of Ssp8 and the tub1-allele of TG130 are associated with low cystnumbers. Two-way ANOVA showed no interaction (P= 0.9).

		Ssp8 tub1	speg	total
TG130	tub1	2.07 (5)	1.76 (24)	1.81 (29)
	tub2	2.24 (19)	1.95 (29)	2.06 (48)
	total	2.20 (24)	1.86 (53)	1.97 (77)

Table 4. Intralocus interaction at the Ac15-7 locus linked to the *Gro*1.2 locus conferring resistance to *G. rostochiensis* pathotype Ro1 on chromosome *10*. The ^{10}log(x+1) transformed NC that were found in each marker-genotype class is given together with the number of plants (in parenthesis). The speg-allele of Ac15-7 is known to be associated with low cystnumbers.

Ac15-7	speg/tub2	speg/tub1+ tub2/tub1	tub1/tub1
	1.74 (17)	1.94 (32)	2.12 (24)

Marker locus Ac15-7 was heterozygous in both parents, with two identical alleles yielding an F2 type segregation. The three marker genotype classes are shown in Table 4. These results confirm that the speg-allele of marker Ac15-7 is linked to a QTL conferring resistance to *G. rostochiensis* pathotype Ro1.

Three way ANOVA was performed to determine the interlocus interaction of the two QTLs, including three alleles, involved in resistance to *G. rostochiensis* pathotype

Ro1. In Table 5 the marker-genotype classes of the resistance linked alleles of Ac15-7, TG130 and Ssp8 are shown and indicate that all effects between the alleles were additive as no significant interaction was found.

Table 5. Three-way table of the markers Ac7, Ssp8 and TG130 linked to QTLs involved in resistance to *G. rostochiensis* pathotype Ro1. The ^{10}log(x+1) transformed NC that were found in each marker-genotype class is given together with the number of plants (in parenthesis). The speg -allele of Ac15-7, speg-allele of Ssp8 and the tub1-allele of TG130 are associated with low cyst numbers. No interlocus interactions were found after ANOVA (P value > 0.2 for all possible combinations).

Ssp8	TG130	speg/tub2	Ac15-7 speg/tub1+ tub2/tub1	tub1/tub1
tub1	tub1	1.72 (2)	- (0)	2.31 (3)
	tub2	2.00 (2)	2.11 (8)	2.45 (7)
speg	tub1	1.67 (7)	1.79 (10)	1.72 (6)
	tub2	1.74 (6)	1.94 (14)	2.06 (8)

Mapping analysis of NT revealed only one QTL on chromosome *4*. The two-way table showing the intralocus interactions at the Ssp47 marker, revealed that the speg-allele was associated with high tuber numbers (Table 6), again no interaction was found after two-way ANOVA.

Table 6. Intralocus interaction of marker Ssp47 linked to a QTL involved in NT. The square root transformed NT that were found in each marker-genotype class is given together with the number of plants (in parenthesis). The speg-allele is associated with high NT. Two-way ANOVA showed no interaction.

Ssp47	tub1	speg
tub1	1.02 (1)	2.90 (26)
tub2	2.26 (21)	2.97 (21)

QTLs with large effects were found for RD. For both loci on chromosome *2* and *6*, segregation of the RFLP alleles was only found for the *S. spegazzinii* parent, so the intralocus interactions could not be determined. Interlocus interaction was determined with two-way ANOVA and the two-way table of TG34 and TG118 is shown in Table 7. A significant interaction was found, indicating epistasis.

Table 7. Interlocus interactions of markers TG34 and TG118, segregating from parent F1-38, both linked to QTLs involved in RD. Two-way ANOVA yielded a significant interaction term (P=0.0015). The phenotypic value for RD that was found in each marker genotype class is given together with the number of plants (in parenthesis).

		TG34		
		tub	speg	total
TG118	tub	1.81 (23)	1.79 (16)	1.80 (39)
	speg	1.14 (18)	1.93 (12)	1.46 (30)
	total	1.52 (41)	1.85 (28)	1.65 (69)

DISCUSSION

Analysis of the quantitative traits

The phenotypic values of the parents are very similar in four out of the nine frequency distributions as can be seen in Figure 1. The phenotypic values of the progeny go beyond the parental values in almost all of the frequency distributions shown. This transgressive segregation implies that QTL alleles, with a positive as well as negative effect are to be expected from both parents.

QTL mapping

Qualitative resistance loci to *G. rostochiensis* pathotype Ro1 have been mapped on chromosome *5* and on chromosome *7*, i.e., the *H1* locus (Pineda et al. 1993; Gebhardt et al. 1993) and the *Gro1* locus (Barone et al. 1990) respectively. Quantitative resistance loci to *G. rostochiensis*, *Gro1.2* and *Gro1.3* loci, as well as *G. pallida*, *Gpa*, have been mapped on chromosome *10*, *11* and *5* respectively, by Kreike et al. (1993 and 1994). The QTLs for both *Globodera* species have been determined in an F1 population derived from a *S. tuberosum* x *S. spegazzinii* cross. In the QTL analysis described in this paper we used a BC population to *S. tuberosum* to confirm and fine map the *G. rostochiensis* QTLs and locate minor QTLs involved in *G. pallida* resistance.

The presence of the *Gro1.2* locus was confirmed with the BC population and located more proximal on chromosome *10* near marker Ac15-7. The explained variance increased thereby from 7 (Kreike et al. 1993) to 19.5%. This increase can conceivably be attributed to a closer linkage between QTL and nearest marker. It is even possible that the tub2-allele, which was identical to the speg-allele, also contributed to the resistance. Kruskal-Wallis tests of another segregating marker of parent Stub, Ssp106, revealed in the F1 as well as the BC population a P-value of 0.09. This could point at the presence of

a QTL at a large distance. If the tub2-allele of parent Stub is also contributing to the resistance than the allele effects of the tub2-allele and the speg-allele are additive (Table 4). Furthermore, a decrease in the environmental variance can contribute to a higher explained variance at marker Ac15-7 as well. Besides, the estimation of the explained variance is ambiguous and variations are possible.

The *Gro*1.3 locus could not be identified in the BC population, although RFLP analysis of the F1 progeny indicated its presence in the F1-38 genotype. However, a new QTL could be located on chromosome *3*, the *Gro*1.4 locus, that was not detected in the F1 population. The QTL-allele conferring resistance was presumably present in *S. spegazzinii* in a homozygous state and could therefore only be revealed in a BC or F2 population and not in an F1 population. However, the resistance allele from the Stub parent might have been detected in the F1 population if more plants would have been used for QTL mapping. Only 57 plants were used in the F1 population and 80 in BC population. Interestingly, the *Gro*1.4 locus was associated with markers segregating from both parents, indicating the existence of multiple alleles at this locus. Multiple alleles have also been revealed at the *Ro* locus involved in tubershape (Van Eck et al. 1994).

RFLP analyses of the F1 population showed that genotype F1-38 was homozygous susceptible at the major *Gpa* locus. Therefore, we aimed for mapping minor QTLs, either from F1-38 or *S. tuberosum*, that possibly were involved in resistance to *G. pallida*. However no QTLs were found.

The amount of the total variation that can be explained by the mapped QTLs is for NC-Ro1 63.5%, NC-Pa2 and NC-Pa3 0%, NT 16% and RD 73%. If these figures are compared to the heritabilities of the quantitative triats (Table 1a+b) it can be noted that for NC-Ro1 and RD almost all genetic variation can be characterised with the found QTLs and additional major QTLs involved in either of these traits are not expected. For NC-Pa2, NC-Pa3 and NT only little of the genetic variation can be explained with the mapped QTLs. Perhaps only minor QTLs are involved in these traits that could not be detected with this experimental design, e.g. number of plants or measurement of the quantitative data. Another explanation can be that the Stub linkage map is not saturated enough, leaving QTLs undetected.

MapQTL (JW Van Ooijen, pers comm.) was used in this study for the localisation of the QTLs. Due to heterozygosity in both parents, RFLP linkage maps could be constructed of F1-38 as well as Stub, but only BC-type segregating markers were used for interval mapping, since they outnumbered the F2-type segregating markers (Figure 2).

At this moment the program is being adjusted for the analysis of non-inbred parents which permits the analysis of the segregation of all alleles at one locus (e.g. from both parents) simultaneously (C. Maliepaard, pers. comm.).

Analysis of intra- and interlocus interaction

Gene action as studied by Edwards et al. (1987) and Stuber et al. (1992) refers to the gene (allele) dosis effect and has mainly been studied in F2 populations of inbreeding species. In both cases QTLs in maize were examined and overdominance was frequently found. In this research a BC population of an outbreeding species is used. A gene dosis effect can only be studied for the allele shared by the two parents (tub1-allele), while the other alleles can also contribute to the trait and interact. Since these interactions are in most cases not based on gene (allele) dosis effects of identical alleles, we preferred to use the term intralocus interactions rather than gene action.

Interlocus interaction or epistasis is a kind of gene interaction whereby one gene interferes with the phenotypic expression of another non-allelic gene. Research into epistasis has also been performed primarily in F2 populations of inbreeding species. Paterson et al. (1991) localised QTLs involved in fruit size, soluble solids concentration and fruit pH in tomato but did not find interlocus interactions, which indicated that the allele effects of the different QTLs were additive. Fatokun et al. (1992), however, found two QTLs involved in seed weight in cowpea that did show a significant interaction.

In the BC population, we did not detect epistasis between QTLs involved in resistance to *G. rostochiensis* pathotype Ro1. For the QTLs involved in RD on chromosome *2* and *6* epistasis was found. Unfortunately, we could not determine the intralocus interactions for both loci, so that a positive or negative contribution of either of the alleles to RD could not be assesed. For a more accurate determination of the interlocus interactions very large populations are needed.

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CHAPTER 6

PROTOCOL:

Non-radioactive detection of single-copy DNA-DNA hybrids.

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ABSTRACT

A new procedure for non-radioactive detection of single-copy DNA-DNA hybrids combines an existing non-radioactive labeling and detection kit with a new substrate AMPPD for the enzyme alkaline phosphatase. The main advantages of this procedure are the possibility to reuse the blots easily and the much shorter detection time compared to radioactive detection methods.

INTRODUCTION

Recently, non-radioactive methods of detecting DNA-DNA hybrids have gained much interest with the improvement of labeling and signal detection. Generally they are based on labeling the DNA with a hapten, *e.g.*, biotin or digoxygenin, for which strong ligands are well characterized (streptavidin or a specific antibody, respectively). These ligands are conjugated to an enzyme such as peroxidase or AP, which converts a substrate, *e.g.*, BCIP and NBT into a colored precipitate on the blot. For full-scale application of non-radioactive techniques two important conditions are necessary: the sensitivity must be equal to radioactive detection and repeated reuse of the filters must be possible. The first condition could be met by the BCIP/NBT system using optimized protocols, but removal of the colored precipitate proved to be difficult. Further advantages of the non-radioactive detection methods are clear. The procedures are less hazardous and signal detection is often much quicker. Even the costs of the non-radioactive detection system, another criterion by which procedures are judged, are not higher than that of ^{32}P methods.

Here we present a step-by-step protocol for the non-radioactive detection of DNA-DNA hybrids using a new substrate for the enzyme AP, which emits light rather than producing a colored precipitate on the blot (Bronstein and McGrath (1989)). The detection of the light signal, called luminography, is carried out with X- ray film in a manner similar to that for the detection of radioactivity. As a consequence, normal stripping procedures for removing the DNA probe allow the blot to be used again. The protocol makes use of a random primer labeling kit, an antibody against digoxygenin linked to AP, a blocking reagent (Boehringer Mannheim, Germany) and the AP substrate AMPPD (Tropix, Bedford, Massachusetts). The protocol also describes a blotting procedure combined with UV crosslinking that yields an improved signal to noise ratio (Allefs et al. 1990).

PROCEDURE

Solutions required:

AMPPD solution: 10 µl AMPPD per ml buffer 3; final AMPPD concentration is 0.26 mM

Anti-dig solution: 3 µl anti-dig per 15 ml buffer 2 (dilution 1:5000)

Buffer 1: 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl

Buffer 2: Buffer 1 plus 0.5% blocking reagent, made 1 hour in advance and heated to 50 to 70°C in order to dissolve the blocking reagent

Buffer 3: 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2$

dNTPs:10 times concentrated mixture containing: 1 mM dATP, 1 mM dCTP, 1mM dGTP, 0.65 mM dTTP, 0.35 mM digoxygenin-dUTP, pH 6.5 (Boehringer Mannheim)

Hexanucleotide mixture: 10 times concentrated hexanucleotide reaction mixture (Boehringer Mannheim)

Hybridization mixture[2]: prehybridization mixture with 40 to 50 ng digoxygenin-dUTP labeled probe per ml

Klenow polymerase: 2 U/µl (Boehringer Mannheim)

Prehybridization mixture: 5X SSC, 0.5% blocking reagent, 0.1% N-lauroylsarcosine, 0.02% SDS

20 x SSC: 3 M NaCl, 0.3 M Na citrate, pH 7.2

TE: 10 mM Tris, 1 mM EDTA, pH 8.0

Transfer solution: 1 M NH_4Ac and 20 mM NaOH[3]

Southern blotting

- After electrophoresis, incubate the gel 5 min in 0.25 M HCL to depurinate the DNA.
- Incubate gel twice for 15 min each time in a mixture of 0.5 M NaOH and 1.5 M NaCl to denature the DNA.
- Incubate gel twice for 10 min each time in the transfer solution[3].
- Cut the membrane to the same size as the gel and incubate it 5 min in water, then 10 min in transfer solution[3].
- Wet the Whatman (3MM) filters in transfer solution[3].
- Build a transfer stack as shown in Figure 1.
- After blotting for at least four hours, the membrane is air-dried overnight at room temperature, and the DNA is UV-crosslinked to the membrane by exposing the membrane, DNA face up, to a 30-W germicidal lamp (Philips TUV)[4] for 1 min at a distance of 40 cm.

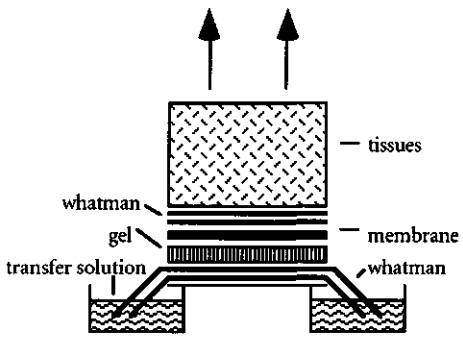

Figure 1. Transfer system for capillary blotting. A glassplate and a weight of one to two g per cm^2 gel are placed on top of the tissues.

Prehybridization

- Wet the membrane[5] in 2X SSC for a few seconds.
- Seal the membrane in a plastic bag[6] with 20 ml prehybridization mixture per 100 cm^2 membrane.
- Incubate for at least one hour at 65°C in a shaking waterbath.

Labeling of the probe

Inserts are isolated from plasmid DNA. Immediately before use the insert DNA is denatured by heating to 95°C for 10 min and cooling quickly on ice. The following solutions are pipetted into a 1.5-ml eppendorf tube:

15 µl insert DNA (10 ng to 3 µg), freshly denatured
2 µl hexanucleotide mixture
2 µl dNTPs (with digoxygenin-dUTP)
1 µl Klenow polymerase

- Mix and incubate overnight at 37°C.
- Precipitate the DNA by adding 1/10 volume of 3M NaAc and 2 vol 96% EtOH (w/v).
- Place at -20°C for 30 min.
- Centrifuge for 15 min at 12,000 rpm and wash the pellet with 70% EtOH (w/v).
- Dry and dissolve the pellet in 50 µl TE[7].

Hybridization

- Discard the prehybridization mixture and add 4 ml hybridization mixture per 100 cm^2 membrane.
- Incubate overnight at 65°C in a shaking waterbath.

Washing

- Pour off the hybridization mixture and wash the membrane four times for 2 min each in 2X SSC, 0.1% SDS (2 ml/cm^2), at room temperature.
- Wash four times for 2 min each in 0.2X SSC, 0.1% SDS (1ml/cm^2), at room temperature.
- Wash twice for 15 min each in 0.2X SSC, 0.1% SDS (1ml/cm^2), at 65°C.

Signal detection[8]

All steps during signal detection are performed at room temperature.

- Rinse the membrane in buffer 1 and seal it in a plastic bag with 10 ml buffer 2 per 100 cm^2 membrane.
- Incubate on a shaker for 30 min.
- Discard the buffer and add 15 ml of fresh anti-dig solution per 100 cm^2.
- Incubate on a shaker for 30 min.

- Discard anti-dig solution and wash the membrane thoroughly three times for 10 min each in buffer 2 (1ml/cm^2) with vigorous shaking. Then wash three times for 10 min each in buffer 1 (1ml/cm^2) with vigorous shaking. Incubate the membrane 5 min in buffer 3 (1ml/cm^2).
- Seal the membrane in a plastic bag with 10 ml AMPPD solution for each 100 cm^2 membrane.
- Incubate on a shaker for 20 min.
- Pour off AMPPD solution and reseal the bag.
- For luminography, put a film on top of the bag containig the wet membrane and place in a casette. Develop the film after 5 to 30 min for detection of multicopy DNA or 30 to 120 min for single copy detection[8,9].

Stripping

After signal detection, rinse the membrane in 2X SSC and strip the probe as follows;

- Incubate 10 min with a mixture of 0.2 M NaOH and 0.1% SDS (1ml/cm^2) at 37°C.
- Rinse the membrane in TE.

Notes

1. The AMPPD solution can be stored at 4°C for a month and reused eight to ten times.
2. The hybridization mixture can be stored at -20°C and reused five to ten times.
3. The addition of alkali to these solutions is optional.
4. If other membranes or UV-sources are used, the optimal UV dose for crosslinking has to be determined experimentally.
5. Do not allow the membrane to dry during any step of the protocol or between consecutive hybridizations.
6. Avoid air bubbles in the plastic bag at the prehybridization, hybridization and detection steps.
7. The labeled probe can be stored for at least six months at -20°C. Prior to hybridization the probe has to be denatured by heating for 10 min at 95°C and cooled quickly on ice.
8. Signal detection can continue for at least eight hours without a significant decrease in signal.
9. The resolution of DNA fragments with similar mobilities will decrease with longer exposure times as with radioactive signal detection.

RESULTS AND DISCUSSION

The protocol described above is for genomic potato DNA digested with a restriction endonuclease. Ten to twenty μg DNA are routinely loaded per lane. For

detection of single-copy sequences, tomato probes from Dr. S. Tanksley (Ithaca, New York) have been used (Fig. 2). With other plant species and probes, *e.g.* sugarbeet, maize and wheat, low- to single-copy DNA has been detected using this protocol. This new method in our laboratory is just as sensitive as standard radioactive methods. The blots can also be reused eight to ten times. The advantages of this method over radioactive methods are now clear: the hybridization mixture can be reused several times in a period of at least one year and the time required for detection is much shorter. An exposure time of two hours is normally sufficient for the detection of single-copy signals, but exposure times of up to 24 hours may be used.

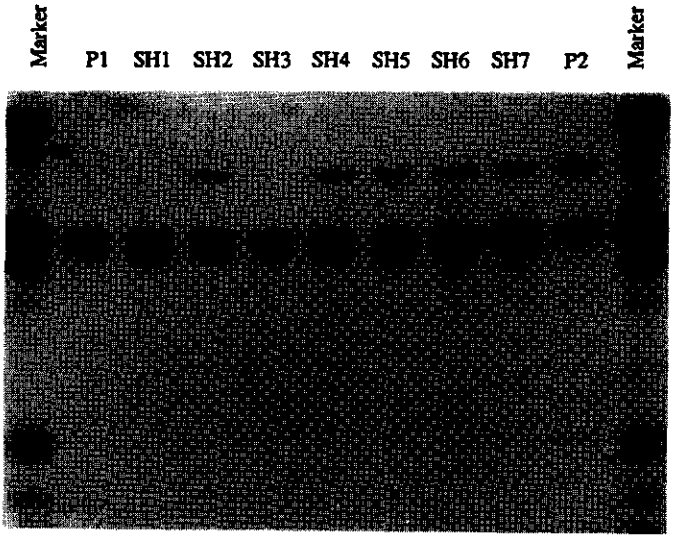

Figure 2. Luminograph showing of single-copy detection. Restriction fragment lenght polymorphism of genomic DNA of potato, digested with *Hin*dIII, of seven somatic hybrids (SH1 to 7) and their parents (P1 and 2) after hybridization with tomato clone TG 42 from Dr. S. Tanksley.

It should be noted that the stringency of the washing procedure may have to be adjusted to achieve optimal results for other conditions because it depends on homology of the probe DNA to the target DNA and whether the target DNA is single- or multicopy. Another factor that affects the strength of the signal is the amount of target DNA on the blot (see "Trouble Shooting").

The optimal UV dose for crosslinking in this procedure depends on two factors: the distance from the membrane to the UV lamp, and the exposure time of the blot. The

optimal UV dose can be determined with a gel with 10 identical lanes containing 1 ng phage λ DNA (Promega) digested with *Hin*dIII and *Eco*RI (Boehringer). After blotting as described above, we exposed the lanes to UV at a fixed distance for 1 to 10 min. After hybridization with labeled λ DNA, washing and detection, the best illumination time at that distance could be easily determined (Allefs et al., 1990).

We have only used capillary blotting in our system and do not know whether electro- or vacuum-blotting might affect the detection of signals.

Several membranes can be used with this protocol; we recommend Hybond N, Hybond N Plus (both Amersham), Duralon (Stratagene) and Genescreen (NEN) membranes. One cannot use a nitrocellulose membrane, however, because AMPPD does not give a signal on this kind of membrane. Genescrene-Plus membrane (NEN) gives a high background. Also PVDF membranes (Millipore) are not suitable for this detection system.

We used the following films for luminography: X-OmatS (Kodak), XR (Fuji) and ECL hyperfilm (Amersham), but no difficulties are expected when other films are used.

Until now the blots are sealed in plastic bags, which is very time-consuming. The use of a hybridization oven in which many blots are hybridized simultaneously is under investigation. The protocol so far can deal with 20 hybridizations in one week with five blots, which are used every day. After washing, detection and luminography the blots are stripped and used the same afternoon in new hybridizations.

TROUBLE SHOOTING

High noise:

- Lower probe concentration in the hybridization mixture.
- Purify the probe DNA (Gene Clean kit II, Bio 101).
- Extend washing procedures with buffer 2 and buffer 1 after antibody treatment.
- Increase the blocking reagent concentration to 1%.

Low signal:

- Increase target DNA concentration on the blot.
- Increase probe concentration in the hybridization mixture.
- Lower stringency during washings after hybridization.

REFERENCES

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CHAPTER 7

DISCUSSION

The construction of an RFLP linkage map and the localisation and characterisation of quantitative trait loci in potato have been described in Chapter 2, 3, 4 and 5. In these successive chapters remarks were made regarding the materials and methods that were used and the results were discussed in relation to those obtained by others. This chapter will pay attention to other related features that were not mentioned in the previous chapters, like linkage mapping in outbreeding species, polyploid and QTL mapping, the relation between qualitative and quantitative traits and comparative mapping. Further research strategies such as fine mapping of QTLs for marker assisted selection and the eventual cloning of a QTL will be described. Finally some comments are given on the classification scheme that is used to differentiate several pathotypes within the potato cyst-nematode species *G. rostochiensis* and *G. pallida* and suggestions are forwarded to improve this scheme.

Linkage mapping in outbreeding species

Most procedures for linkage analysis and map construction that are described in literature use inbreds (fully homozygous genotypes) as parents (see Chapter 1). If mapping is carried out with outbreeding species (heterozygous parents), like potato, alfalfa or apple, the existing procedures can be used after proper adjustments of the original data sets. Sometimes the segregation of alleles from one parent only are used, thus treating the F1 population as a BC population (Bonierbale et al. 1988). In other cases (Chapter 2, 3 and 4; Hemmat et al. 1994), the F1 populations have been treated as two BC populations by scoring the segregating alleles of both parents separately. Gebhardt et al (1989) also used the segregation data of alleles that were shared between the parents yielding a dominant F2 segregation (3:1). For our mapping experiments the computer program Joinmap was used, which is primarily developed for inbreds. A modified version of JoinMap, for the construction of linkage maps for outbreeding

species that takes into account the segregation of all alleles at a locus, i.e. from both parents simultaneously, is in progress (P. Stam, pers. comm.).

Polyploid mapping

S. tuberosum is an autopolyploid species with 4 chromosome sets that are all capable of pairing and recombining with each other. If a genetic linkage map was constructed from crosses between tetraploids, 6 allelic combinations are possible per locus in the gametes. Therefore, it is obvious that the determination of crossing-over frequency in such crosses is a complicated task. To circumvent this, the RFLP map described in Chapter 2 has been constructed with diploid *S. tuberosum* genotypes and wild *Solanum* species, since mapping with a diploid population is far more easier. Recently, methods have been described by Wu et al. (1992) that allow mapping in polyploid species. They suggested to follow the distinct chromosomes with molecular markers, provided that they posses a unique allelic fragment, also called a single-dose restriction fragment (SDRF). These SDRFs can be used for linkage studies, thus enabling the construction of a linkage map and ultimately localisation of interesting traits in polyploid organisms which otherwise could not be used for these analyses.

QTL mapping

The program that has been used for QTL mapping in Chapter 4 and 5, MapQTL (JW Van Ooijen, pers comm.), is also primarily developed for inbreeding species, and as described above, minor modifications of the segregation data are neccesary if heterozygous parental genotypes are used. A new version of this program is being developed to deal with outbreeding species as well (C. Maliepaard, pers. comm.).

The use of DNA pools, also called bulked segregant analysis (BSA), based on phenotypic information has been very succesfull for mapping qualitative trait loci (Michelmore et al. 1991). Wang and Paterson (1994) investigated whether this method could be used for QTL mapping as well. In general they found that BSA might be succesfull in detecting QTLs with very large effects, but most likely the majority of QTLs affecting a complex trait would not be identified this way. However, if a priori information exists (from RFLP mapping), DNA pools can be made to find new markers for the region known to contain a QTL (see section Fine Mapping).

Also progress is being made with respect to the statistical methods for QTL detection and mapping. New models, based on interval mapping, have been proposed to

(a) deal with non-normally distributed traits, (b) decrease the environmental variation, and (c) reduce genotypic variation by taking into account the effects of two or more QTLs at once, thereby increasing the statistical power and the precision of the QTL mapping. (Jansen 1992 and 1993; Jansen and Stam 1994). Haley et al. (1994) developed a Least Squares method for the localisation of QTLs in outbreeding species. In this approach information from several linked markers is used. This procedure increases the sensitivity of the test statistics and the power of the detection of QTLs, compared to the use of single markers or markers flanking an interval. This method has recently also been applied by Andersson et al. (1994) for the mapping of QTLs involved in growth and fatness in pigs.

Relationship between qualitative and quantitative traits: multiallelism

Robertson (1985) was the first to suggest that alleles for qualitative mutants could be simply loss-of-function alleles at the same loci underlying quantitative variation. If a gene contributing to quantitative variation is allelic to a gene controlling qualitative variation, then these genes should map to the same locus. Since more and more QTLs have been mapped this hypothesis has been tested. Beavis et al. (1991) mapped plant height, a quantitative character, in maize and compared the map positions of the detected QTLs with previously known positions of qualitative variations of the same trait (plant height mutants). The results showed a general concordance in map position of QTLs and major genes affecting plant height. Another example is found by Goldman et al (1993), who described the mapping of QTLs involved in protein and starch content near the *Shrunken-2* (*SH-2*) locus in maize. The *SH-2* locus contains a structural gene encoding the major subunit of the starch synthase enzyme ADP-glucose pyrophosphorylase. Also a QTL involved in field resistance to *Phytophthora infestans* in potato mapped to the same chromosomal region as the *R1* locus (Leonards-Schipper et al. 1994). The *R1* locus is a major locus involved in race-specific resistance to *P. infestans* (Leonards-Schippers et al. 1992; El-Kharbothly et al. 1994) on chromosome *5*.

The existence of multiple alleles at one locus was first demonstrated by Van Eck et al. (1994) for tuber shape. Also for other quantitative traits in potato, i.e., earliness and flesh colour, multiple alleles have been found (HJ Van Eck, pers comm.). The presence of multiple alleles at the *Gro*1.4 locus on potato chromsome *3* is described in Chapter 5. This QTL is involved in resistance to *G. rostochiensis* pathotype Ro1.

Comparative mapping

For some related species comparative linkage maps have been made using a common set of probes. Examples are tomato-potato-pepper (Bonierbale et al. 1988 and Tanksley et al. 1988), *Brassica campestris-B. oleracea* (Slocum 1989), maize-sorghum-rice-wheat-barley-rye (Ahn et al. 1993; Ahn and Tanksley 1993; Hulbert et al. 1990; Moore et al. 1993; Whitkus et al. 1992), and man-cattle-mice (Davisson et al. 1991; Womack 1990). Comparison of these maps show that the genome organisation of these more or less related species is very similar and that the order of the loci along a chromosomal segment is often identical. Differences between the genomes can be attributed to duplications, rearrangements or inversions of certain parts of the chromosomes.

To a lesser degree, comparative mapping may also be possible with regard to QTLs. In a recent study Paterson et al. (1990) compared the locations of QTLs in two different wild species of tomato, *L. chmielewskii* and *L. cheesmanii*, which are only distantly related (Miller and Tanksley 1991) but are similar in having very small fruit with high soluble solids content. About half of the QTLs that were mapped in the two species fell at similar chromosomal locations, suggesting that comparable genetic factors influence quantitative traits in these two distantly related species. Another example is described by Fatokun et al. (1992) who studied QTLs for seed weight in the species cowpea and mungbean, *Vigna unguiculata* and *V. radiata* resp.. The most significant QTL determining seed weight mapped to the same chromosomal locus in the genomes of both species. Both studies show that it might be possible to predict the positions of important QTLs (e.g. for growth rates or yield) in one species based on mapping studies from another species. This seems especialy interesting for economically important crops that have too complex genomes for molecular genetic analysis. A related model species, with simpler genome composition can than be used to genetically resolve complex traits.

Further research

Now QTLs, conferring resistance against *G. rostochiensis* or *G. pallida*, have been mapped and characterised, an experimental design can be made for using these QTLs in plant breeding or for investigating the structure and function of the genes itself. QTLs can be introduced in a crop with the straightforward approach of marker assisted selection (MAS). For structure/function research QTLs have to be isolated which can be carried out following two different strategies; map-based cloning or transposon tagging.

Before either MAS or the isolation of a QTL can be initiated, fine mapping of the QTL region with closely linked markers in order to locate the QTL more precisely, will however be necessary.

Fine mapping

The precise localisation of a QTL requires many markers located in the chromosomal region comprising the QTL. The construction of high density molecular linkage maps of the entire genome, consisting of RFLP, RAPD, STS or AFLP markers, as has been done for tomato and potato (Tanksley et al. 1992) will provide such large numbers of markers. Additional markers can also be identified for just the small region of the genome in which the QTL is located. These markers can be obtained by pooling DNA (BSA) based on prior genotypic information and subsequently performing RAPD or AFLP analysis on these pools. Using RAPD analysis, fine maps have been made of QTLs affecting fruit ripening and jointless stem in tomato (Giovannoni et al. 1991) and of QTLs involved in nematode resistance in soybean (Ferreira et al. 1994). BSA combined with AFLP analysis has also been used for fine mapping of the *R1* locus of potato (Meksem et al. 1994).

Paterson et al. (1990) described yet another method, based on substitution mapping, to obtain detailed information on the location of a QTL. The resolution of this approach depends on the number and spacing of genetic markers available to distinguish overlapping recombinant segments. This method (also) determines the presence of one or multiple loci, each of them contributing to the same quantitative trait, in a region displaying QTL activity. It is also possible to resolve the pleiotropic effect of a QTL into the presence of several closely linked genes, each of them influencing different traits (Paterson et al. 1990). If a postulated QTL is the result of the action of two or more linked genes, it might be possible to separate those by crossing-over and measure their individual contribution to the quantitative trait(s).

Marker assisted selection (MAS)

With molecular markers surrounding a QTL, marker assisted selection (MAS), can be employed to examine whether or not introgression of a desired trait has happened. This procedure might be much faster and simpler than performing complicated tests to establish the phenotypic value of a plant. However, tight linkage of marker and QTL is required to diminish the chance of recombination. Most QTLs are mapped within a

resolution of 10-20 cM. Such a 20 cM region might include genes with undesirable effects, which precede agricultural utility of the resident favourable genes (Tanksley and Hewitt 1988; Paterson et al. 1990).

Map-based cloning and transposon tagging.

Because the biochemical function of genes which embody a quantitative trait are usually not known, molecular cloning of a QTL would give more insight into its structure and function. There are two methods to clone a gene with an unknown gene product, map-based cloning and transposon tagging. So far map-based cloning has only been successful in a few cases of single genes with striking effects (Royer-Pokora et al. 1986; Rommens et al. 1989; Gessler et al. 1990). The first plant genes that were cloned this way were from *Arabidopsis* (Giraudat et al. 1992; Arondel et al. 1992). Recently also resistance genes against *Pseudomonas syringae* pv tomato (*Pto*) from *Lycopersicon esculentum* (Martin et al. 1993) and *Pseudomonas syringae* (*RPS2*) from *Arabidopsis* (Staskawitcz et al. 1994) have been cloned.

Another strategy for isolating genes with unknown gene product but with an easily recognizable mutant phenotype is transposon tagging. The succes of this technique has been demonstrated in species like maize and *Antirrhinum* (Wienand and Saedler 1987) as well as in heterologous species like *Arabidopsis* (Aarts et al. 1993; Bancroft et al. 1993) and petunia (Chuck et al. 1993). Recently, the *Cf*-9 resistance gene conferring resistance to *Cladosporium fulvum* in tomato has been isolated by targeted transposon tagging (Jones et al. 1994). The transposon was first delivered close to the locus (targeting) because high frequencies of insertion at linked loci can be obtained, since Ac transposes preferentially to closely linked chromosomal positions (Jones et al. 1990; Jacobs et al. 1994). Experiments for targeted transposon tagging in potato are in development (Pereira et al. 1992).

The pathotype classification scheme

The identification of pathotypes within the species *G. rostochiensis* and *G. pallida* has been a matter of much debate. Already in 1964 Dunnet proposed a nomenclature based on major genes, similar to that for *Phytophthora infestans*. However the knowledge of the plant genes controlling the resistance and the genes of the potato cyst-nematode involved in the interaction was inadequate at that time. It took till 1977 when Kort et al. presented an international pathotype scheme, based on the British and Dutch

classification schemes. This pathotype scheme does not require a detailed knowledge of the genetics of host and pathogen, and does not make assumptions about the biology of the potato cyst-nematodes. Pathotypes have been defined in this scheme as variants of a potato cyst-nematode species which differ from each other by their (in)ability to multiply on particular potato genotypes known as differential hosts (Robinson 1969). In this scheme they introduced the Pf/Pi ratio (see Chapter 1, section potato cyst-nematodes). If this ration was ≤ 1.0 this was an indication for resistance and if it was > 1 for susceptibility. Prior to 1977 ratings were made relative to the susceptible standard. Together with the ignorance of the genetic base on the plant side as well as on the nematode side, is the arbitrary threshold of a Pf/Pi ratio of 1 for defining resistance against susceptibility, the major drawback of this scheme.

It is now known that the resistance of one of the differentials, *S. tuberosum* ssp *andigena* CPC1673, is due to one major locus, *H1*, (Toxopeus and Huijsman 1953; Dunnet 1963) which is located on chromosome 5 of potato (Gebhardt et al. 1993 and Pineda et al. 1993). The resistance of the differentials derived from *S. vernei* appeared to be polygenic (Goffart and Ross 1954) and due to a complex of major and minor genes (Ross 1969; Huijsman 1974). Also both the resistance from *S. multidissectum* (Dunnet 1962) as well as from *S. kurtzianum* (Huijsman 1960) are supposed to be due to major genes, resp. the *H2* and A and B loci. The existence of these major loci has however not been confirmed by RFLP mapping analysis.

Trudgill (1985) has criticized the arbitrary threshold of Pf/Pi < 1 for resistance in the pathotype scheme, because factors like initial population density, vigour of plant growth and viability of the nematode influence the Pf/Pi measurements, so they cannot be used as a reliable measurement for nematode virulence or plant resistance. The discrimination between *G. pallida* pathotype Pa2 and Pa3 for instance is based on differential VTN62-33-3. This discrimination is doubtful since Pa2 is denominated avirulent with a Pf/Pi of 1.1-1.2 and Pa3 is denominated virulent with a Pf/Pi of 6-8, while on the susceptible standard Pa3 has a Pf/Pi of 10-30 (Trudgill 1985; Janssen 1990; Nijboer and Parlevliet 1990).

Another example is the discrimination by *S. multidissectum* of Pa1 from the Pa2/3 complex. *S. multidissectum* (*H2*) possesses partial resistance to Pa2 and Pa3 (Pf/Pi 2-5 instead of 10 -30 on susceptible differential (Nijboer and Parlevliet 1990) and therefore the distinction of Pa1 from Pa2/3 is doubtful. The definition of resistance and the subsequent pathotyping based on the Pf/Pi ratio is thus unsatisfactory (Trudgill 1985;

Nijboer and Parlevliet 1990). In terms of its agronomic significance this threshold is a reasonable borderline but biologically it is merely an arbitrary point in a continuum between full resistance (no reproduction at all), and extreme susceptibility (large reproduction).

Not only the lack of genetic knowledge on the resistance in the plant but also of the (a)virulence in the nematodes hampers the discrimination of the potato cyst-nematodes in pathotypes. According to Trudgill (1985) a pathotype should meet the following definition formulated earlier by Anderson and Anderson (1982): "all individuals of a pathotype should have one gene, or group of genes, for (a)virulence, which is common to all individuals of that pathotype and differs from that found in any other pathotype".

The influence of the applied pathotype classification scheme on the mapping of the nematode resistance loci

The research described in this thesis was carried out with the nematode populations Mierenbos A, P2-22 and Coll1077. These can be distinguished into respectively pathotype Ro1, Pa2 and Pa3 with the above discussed pathotype scheme. If the discrimination of the potato cyst-nematodes into pathotypes with this scheme is inadequate, this could have certain implications for the obtained results described in this thesis.

G. rostochienis and *G. pallida* are discriminated morphologicaly and biochemically as two different species (see Introduction). The QTLs, involved in resistance to either one of them, were detected with only one population of *G. rostochiensis* and two populations of *G. pallida*. Therefore caution must be paid to the extrapolation of the resistance spectrum to different nematode populations classified on the differentials as the same (or other) pathotypes. Additional resistance tests have to be carried out with other nematode populations to determine the full spectrum of a resistance locus.

The nematode populations P2-22 and Coll1077 belong to pathotypes Pa2 and Pa3 resp. according to the pathotype scheme. The *Gpa* resistance locus could however not discriminate between the two pathotypes as it conferred resistance to both of them. Additional resistance tests with two other populations, D236 and Rookmaker (pathotype Pa2 and Pa3, resp.) were performed on a small scale and showed that the *Gpa* locus also conferred resistance to these two populations so probably this locus has a broad resistance spectrum. However small differences were observed in the resistance level of the plants that carry the *Gpa* locus after infection with the four different *G. pallida* populations.

These differences might be caused by impurities of the populations, i.e., the population did not exclusively exist of individuals that all carry the (same) avirulence gene. The use if such impure nematode populations might affect the determination of the precise location of the *Gpa* locus and underestimate the calculated effect of this locus.

In conclusion it can be noted that although the pathotype classification scheme does not clearly define the levels of virulence of the nematode populations, it does not appear to affect the obtained mapping results in a significant way.

Suggestions for the classification of pathotypes within the potato cyst-nematode species

It is possible to discriminate nematode populations on the basis of their avirulence gene(s) with the gene pool similarity concept (Bakker 1994). The gene pool similarity concept rests on the hypothesis that in the absence of selection pressure by host resistance genes, degrees of similarity between populations, revealed by molecular techniques, are also reflected in similarity at avirulence loci, including those not yet resolved by current pathotype schemes (Bakker 1987; Bakker et al. 1992; Bakker and Gommers 1989). Two dimensional gel electrophoresishas been used for the assessment of gene pool similarities (Bakker and Bouwman-Smits 1988; Bakker et al. 1992; De Boer et al. 1992). This work indicated that the genetic variation between European populations is predominantly the result of the genetic structures of the primary founders, random genetic drift and gene flow, and not the result of selection for virulent genotypes. This implies that molecular data are valuable indicators for interpopulation variation in avirulence. In other words, identical or closely related populations, as established by molecular techniques, will show resemblance at their avirulence loci.

It is clear from the above mentioned arguments that the pathotype classification of Kort et al. (1977) has no solid biological basis. Fortunately, the knowledge of the genetics of the resistance increased considerably. New resistances has been found which are dominated by major loci. Examples of these are the *Gro*1 locus on chromosome 7 of *S. spegazzinii* which confers resistance to Ro1 and presumably Ro5 (Barone et al. 1990) and the *GroV*1 locus from *S. vernei* which confers resistance to Ro1 and Ro4 (JME Jacobs, pers. comm.). The latter is mapped in the same region on chromosome 5 as the *H*1 resistance locus from *S. tuberosum* ssp *andigena*. Also for *G. pallida* two major resistance loci have been described. The first is derived from *S. tuberosum* ssp *andigena* CPC1673 which confers resistance only to the D236 population (Arntzen et al. 1993).

The second locus, *Gpa*, is identified in *S. spegazzinii* and confers resistance to several Pa2 and Pa3 populations and is also located on chromosome 5 (see Chapter 5).

Molecular analysis of nematode populations will also increase our knowledge about the avirulence genes present in nematode populations and together with the renewed insight in the resistance genes in potato, an improved pathotype classification scheme can be constructed based on gene-for-gene relationships of resistance loci and avirulence genes as has been shown for the *H*1-Ro1 system (Janssen 1990; Janssen et al. 1991).

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Summary

Potato cyst-nematodes belong to the major pests of potato (*Solanum tuberosum* ssp *tuberosum*) and cause considerable damage to the potato crop. Two species of potato cyst-nematodes, *Globodera rostochiensis* and *G. pallida*, have been identified and several pathotypes can be distinguished for each species. Resistance against these nematodes has been found in many wild *Solanum* species. These resistances often have a quantitative nature and are presumed to be dominated by several genes, also called quantitative trait loci (QTLs). *S. spegazzinii* accession BCRC 8218 is resistant to both *Globodera* species and the inheritance of these resistances and the number, location and interaction between the QTLs involved in the resistance are described in detail in this thesis.

Molecular techniques, in particular Restriction Fragment Length Polymorphism (RFLP) analysis, were used for localisation of the QTLs on the chromosomes. Chapter 2 describes the construction of an RFLP linkage map of the potato chromosomes. For this map, RFLP markers from a genomic DNA library of *Solanum spegazzinii* were employed and the computer program JoinMap was used to construct the linkage map. In total 106 loci were detected on the 12 chromosomes by the 95 markers resulting in linkage map of 731 cM. With this map the reduced recombination and distorted segregation in an interspecific hybrid between *S. tuberosum* and *S. spegazzinii* (F1-38) was investigated. Gamete selection was mostly responsible for the observed distorted segregation ratios. Selection at the zygote level was directed against homozygous genotypes and was found in a backcross population (F1-38 x *S. tuberosum*) for markers on chromosome *2*, *3* and *4*. Also the recombination frequencies between the male and female linkage map of *S. tuberosum* were compared, which suggested a reduced recombination in the male linkage map.

Chapter 3 describes the mapping of quantitative resistance to the potato cyst-nematode *Globodera rostochiensis* pathotype Ro1 in an F1 population of *S. tuberosum* x *S. spegazzinii*. One hundred and seven RFLP markers were tested in combination with 4 different restriction enzymes for a heterozygous RFLP pattern within *S. spegazzinii*. Only 29 markers were segregating for *S. spegazzinii* alleles in the F1 population and this limited set of markers was used for mapping the resistance trait. Analysis of variance

(ANOVA) was applied to test the association of the RFLP markers with nematode resistance. Two QTLs, *Gro*1.2 and *Gro*1.3, involved in resistance to *Globodera rostochiensis* pathotype Ro1 were identified and mapped on chromosome *10* and *11*, respectively. An interaction between these loci was not found, indicating additivity of the alleles involved in resistance.

Chapter 4 describes the mapping of quantitative resistance to *Globodera pallida* pathotypes Pa2 and Pa3 in an F1 population of *S. tuberosum* x *S. spegazzinii*. Based on the results of the resistance tests, the existence of 2 to 3 major loci involved in resistance was initially predicted. However, only one major locus, *Gpa*, derived from *S. spegazzinii*, was responsible for the high resistance level. This locus was mapped on chromosome *5* and two minor loci were mapped on chromosome *4* and *7*. Additionally, the contribution of the susceptible parent to the nematode resistance was determined and minor loci were postulated on chromosomes *1, 3, 5, 11* and *12*. The discrepancy between the expected number of major loci and the observed number could be explained by the detected distorted segregation on chromosome 5. An RFLP marker closely linked to this resistance locus showed a shortage of plants having the resistance linked allele, hence also the resistance locus was exposed to segregation distortion. These results indicate that a prediction of the genetic constitution of a quantitative trait, solely based on phenotypic observations can lead to erroneous conclusions.

In Chapter 5, a BC population (F1-38 x *S. tuberosum*) was used to confirm the mapped QTLs involved in nematode resistance. Also other quantitative traits like tuber yield and root development were analysed and mapped in this population. Additionally the intra- and interlocus interactions of these QTLs were examined. A complete linkage map was available for both parents and interval mapping could be used for the localisation of the QTLs on the chromosomes. The presence of the *Gro*1.2 locus on chromosome *10* was confirmed and located more precisely. A new resistance locus *Gro*1.4 was identified on chromosome *3*. Both parents had different alleles at the *Gro*1.4 locus that contributed to the resistance trait, indicating multiallelism at this locus. The *Gro*1.3 locus, mapped in the F1 population, could not be located. QTLs that were involved in resistance to either *G. pallida* pathotype Pa2 or Pa3 could not be detected in the BC population. For root development, 2 loci with large effects were mapped on chromosome *2* and *6*, and an epistatic interaction was observed between these two loci. For tuber yield only a minor QTL was found on chromosome *4*.

RFLP analyses are generally carried out with hybridisation and detection techniques that use radio-isotopes (^{32}P). However, during this research I used a non-radioactive method for detection which is described in Chapter 6. This procedure combines an existing non-radioactive labelling and detection kit with a 'new' substrate AMPPD (3-[2'-spiroadamantane]-4-methoxy-4-[3"-phosphoryloxy] phenyl-1,2-dioxetane disodium salt). When this substrate is converted by alkaline phosphatase, light is emitted which can be detected with X-ray films. The main advantages of this protocol are the possibility to reuse the membranes easily, compared to the use of other substrates for non-radioactive detection, and the much shorter detection time, compared to radioactive detection methods.

Related subjects that could not be dealt with in the previous chapters are described in Chapter 7. The subsequent application of the obtained results for marker assisted selection (MAS) or for isolation of the QTLs with map-based cloning or transposon tagging, are indicated. Further the pathotype scheme that is used to discriminate several pathotypes within the species *Globodera rostochiensis* and *G. pallida* is discussed and suggestions are made for the improvement of this classification.

Samenvatting

Het aardappelcysteaaltje is een van de belangrijkste pathogenen van de gecultiveerde aardappel (*Solanum tuberosum* ssp *tuberosum*) en kan voor aanzienlijke opbrengstverliezen zorgen. Er zijn twee soorten aardappelcysteaaltjes bekend, namelijk *Globodera rostochiensis* en *G. pallida*, die elk weer onderverdeeld kunnen worden in een aantal pathotypen. Het is noodzakelijk om resistentie tegen deze nematoden te vinden omdat de gecultiveerde aardappel van zichzelf zeer vatbaar is voor deze pathogenen. Gelukkig zijn er enkele wilde aardappel (*Solanum*) soorten gevonden die wel resistent zijn tegen het aardappelcysteaaltje. Echter, de resistenties zijn veelal kwantitatief van aard en er wordt aangenomen dat er vele genen ('Quantitative Trait Loci' oftewel QTLs) bij betrokken zijn. Dit maakt het overbrengen van deze resistenties via kruising en selectie vanuit de wilde verwanten naar de cultuuraardappel een moeizaam proces.

Moleculaire technieken kunnnen dit proces vereenvoudigen. Met behulp van moleculaire markers zoals Restrictie Fragment Lengte Polymorfismen (RFLPs) kan de overerving van deze complexe resistentie bestudeerd worden en de genen die betroken zijn bij de resistentie op de chromosomen gelocaliseerd. Dit karteren gebeurt door RFLP markers te vinden die gekoppeld zijn aan de resistentiegenen. De plaats van de RFLP markers op de chromosomen is bekend en daarmee tevens de plaats van het resistentiegen. Het opsporen van koppelingen tussen RFLPs en resistentiegenen heeft tot gevolg dat de aanwezigheid van het resistentiegen in een plant eenvoudig is aan te tonen waardoor tijdrovende resistentietoetsen niet meer nodig zijn. Tevens kan het resistentiegen gemakkelijker gevolgd worden in een kruisingsprogramma door te selecteren op genotypen die de gekoppelde RFLP markers bezitten.

Solanum spegazzinii is één van de wilde aardappelsoorten die resistentie tegen beide aardappelcysteaaltjes bezit. In dit proefschrift wordt de overerving van deze resistenties, het aantal genen dat bij de resistenties betrokken is en de localisatie van deze genen op de chromosomen met behulp van RFLPs, beschreven.

In Hoofdstuk 2 wordt de constructie van een RFLP kaart van het aardappelgenoom beschreven. De RFLP kaart werd voornamelijk gemaakt met RFLP

markers die afkomstig zijn van een genomische DNA bank van *S. spegazzinii*. Het computerprogramma Joinmap werd gebruikt om de RFLP kaart te construeren. Deze RFLP kaart is 731 cM lang en bestaat uit 106 loci die verdeelt zijn over de 12 chromosomen. Met deze kaart zijn de verminderde recombinatiefrequentie en de scheve uitsplitsingsverhoudingen in een soortshybride van *S. tuberosum* x *S. spegazzinii* (F1-38) onderzocht. Scheve uitsplitsingsverhoudingen, mogelijk veroorzaakt door selectie op gameetniveau, werden gevonden op alle chromosomen. In de terugkruisingspopulatie (F1-38 x *S. tuberosum*) werd op specifieke plaatsen op chromosoom *2, 3* en *4*, selectie tegen homozygote genotypen waargenomen. Tevens werden de recombinatiefrequenties van de mannelijke en vrouwelijke kaart van *S. tuberosum* met elkaar vergeleken. De mannelijke kaart bleek, net zoals de kaart van de soorthybride, een lagere recombinatie-frequentie te hebben.

In Hoofdstuk 3 wordt onderzoek beschreven dat als doel had de kwantitatief overervende resistentie tegen het aardappelcysteaaltje *Globodera rostochiensis* pathotype Ro1 te localiseren met behulp van RFLPs in een F1 populatie (*S. tuberosum* x *S. spegazzinii*). Honderdenzeven RFLP markers werden in combinatie met 4 restrictieenzymen uitgetest om heterozygotie in *S. spegazzinii* op te sporen. Helaas lukte dit maar voor 29 van de 107 RFLP markers. De localisatie van de resistentie met deze 29 markers gebeurde door middel van variantie analyse (ANOVA). Twee QTLs, *Gro*1.2 en *Gro*1.3, werden gevonden die betrokken waren bij resistentie tegen *G. rostochiensis* pathotype Ro1. Deze loci bleken respectievelijk op chromosoom *10* en *11* te liggen.

In Hoofdstuk 4 wordt onderzoek beschreven waarbij de F1 populatie (*S. tuberosum* x *S. spegazzinii*) gebruikt wordt om resistentiegenen tegen *G. pallida* pathotypen Pa2 and Pa3 te localiseren. De resultaten van de resistentietoets duidden op de aanwezigheid van 2 of 3 hoofdgenen. Er werd echter maar één hoofdgen gevonden, het *Gpa* locus, afkomstig van *S. spegazzinii*, dat verantwoordelijk was voor het hoge resistentieniveau. Dit locus ligt op chromosoom *5*. Verder zijn twee bijgenen, ook afkomstig van *S. spegazzinii*, gelocaliseerd op chromosoom *4* en *7*. Het is mogelijk dat ook bijgenen liggen op chromosoom *1, 3, 5, 11*, of *12*, afkomstig van *S. tuberosum*, die bijdragen aan de resistentie. Het verschil tussen het voorspelde aantal hoofdgenen en het gevonden aantal kan verklaard worden met de waargenomen scheve uitsplitsing op chromosoom *5*. De RFLP marker die vlakbij het resistentie locus ligt heeft in de F1populatie een tekort aan allelen die gekoppeld zijn aan de resistentie. Door de lage aantallen resistente planten werd de suggestie werd gewekt dat er meer hoofdgenen bij

de resistentie betrokken zouden zijn. Dit resultaat laat zien dat het riskant is om alleen op grond van de resistentie toetsen een schatting te maken van het aantal genen dat betrokken is bij de resistentie.

In Hoofdstuk 5 wordt onderzoek beschreven waarbij de terugkruisings populatie (F1-38 x *S. tuberosum*) gebruikt is om kwantitatieve eigenschappen zoals nematodenresistentie, knolopbrengst en wortelontwikkeling te localiseren op de chromosomen door middel van "interval-mapping". De bijdragen van beide ouders aan deze eigenschappen werden onderzocht. Het *Gro*1.2 locus dat betrokken is bij resistentie tegen *G. rostochiensis* pathotype Ro1 werd wederom aangetoond en kon zelfs nauwkeuriger op chromosoom *10* geplaatst worden. De aanwezigheid van resistentie-allelen afkomstig van het *Gro*1.3 locus kon niet worden aangetoond in de BC populatie. Een nieuw resistentielocus *Gro*1.4 werd echter gevonden op chromosoom *3*. Beide ouders bezitten daarnaast andere allelen van dit locus die van invloed zijn op de resistentie. Er werden geen genen gevonden die betrokken waren bij resistentie tegen *G. pallida* pathotype Pa2 of Pa3. Voor wortelontwikkeling werden 2 hoofdgenen gevonden op respectievelijk chromosoom *2* en *6*, die beide een aanzienlijke bijdrage leverden aan deze eigenschap. Voor knolopbrengst kon maar 1 bijgen gevonden worden en wel op chromosoom *4*.

Meestal wordt tijdens RFLP onderzoek gebruik gemaakt van radio-isotopen (^{32}P). Gedurende mijn onderzoek heb ik echter gebruik gemaakt van niet-radioactieve methoden. In Hoofdstuk 6 wordt een methode beschreven waarbij gebruik gemaakt wordt van een nieuw substraat, AMPPD. Dit substraat wordt omgezet door het enzym alkalische fosfatase waarbij licht vrijkomt. Het vrijkomende licht wordt vervolgens gedetecteerd met behulp van een röntgenfilm. Deze methode maakt het gebruik van radio-isotope stoffen voor RFLP analyse overbodig en is sneller vanwege de kortere detectietijd. Een bijkomend voordeel van dit protocol, ten opzichte van andere niet-radioactieve methoden, is dat er geen gekleurd precipitaat op het membraan achterblijft, zodat hergebruik van het membraan mogelijk is.

In het laatste Hoofdstuk, 7, worden enkele onderwerpen behandelt die in de voorgaande hoofdstukken niet ter sprake zijn gekomen, zoals het maken van kaarten met kruisbestuivende gewassen of polyploide soorten, multiallelie, en het vergelijken van RFLP kaarten van nauwverwante soorten. Daarnaast wordt ingegaan op eventuele vervolg experimenten die het gebruik van marker-gestuurde-selectie (MAS) of gen-isolatie mogelijk maken. Vervolgens worden kanttekeningen geplaatst bij de

onderverdeling van de aardappelcysteaaltjes *G. rostochiensis* and *G. pallida* in verschillende pathotypen en worden suggesties gedaan om deze onderverdeling te verbeteren met behulp van de nieuw verkregen inzichten over de resistentiegenen van de plant en (a)virulentiegenen van de aardappelcysteaaltjes.

Nawoord

Na een klein oponthoud is mijn proefschrift afgekomen en tevreden kijk ik terug op de afgelopen 6,5 jaar waar het promotie-onderzoek een groot deel van uitmaakte. Tijdens het onderzoek heb ik een heleboel mensen ontmoet die allemaal aan dit proefschrift hebben bijgedragen en hiervan wil ik er enkele noemen.

Allereerst Lidwien Dellaert, die het AM-project, mede-gefinancierd door de Nederlandse Aardappel Associatie, heeft opgestart en het voorbereidende onderzoek heeft verricht naar de overerving van de resistentie. De resistentietoetsen werden voornamelijk uitgevoerd door Henk Vinke, bijgestaan door Jan Menting. Gelukkig bleef Henk ook na het weggaan van Lidwien betrokken bij het onderzoek en de resistentietoetsen van de F1 en BC populatie zijn grotendeels onder zijn leiding uitgevoerd. Dit gaf mij de gelegenheid om me volledig op het molekulair-biologische deel van het onderzoek te storten.

Ik heb in de 5 jaar die ik op het lab stond drie assistenten en 1 student gehad. Allereerst was er Jacq de Koning. We hebben samen het onderzoek opgestart en de verhuizing van SVP naar ITAL meegemaakt. In deze tijd hebben we heel wat afgepraat. Met Jacq en Jan-Derk, mijn enige student, als 'bodyguards' kon ik veilig door het gebouw lopen. Vervolgens kwam Wendy Oude Breuil. Of er nu blots gedraaid moesten worden of cysten geteld, alles werd even enthousiast en vrolijk gedaan. De laatste assistente was Aukje Kok-Westeneng. Zij heeft even flink de vaart in de nog lopende proeven gezet waardoor alle experimenten die ik in het laatste jaar aangepland had afgekomen zijn. Achteraf had ik mij geen betere assistenten kunnen wensen.

Door de vele fusies van de verschillende instituten ben ik een paar keer van werkplek veranderd wat de continuïteit van het onderzoek niet ten goede kwam. Wel heb ik door die verhuizingen veel mensen binnen het CPRO leren kennen die ook hun invloed hebben gehad op het onderzoek. Ik ben begonnen op het SVP onder begeleiding van Frans Krens. Op het lab heb ik toen samengewerkt met Gerard Rouwendal, John Jacobs, Sjefke Allefs en Elma Salentijn. De grote vrijheid om het onderzoek uit te voeren zoals ik wilde en tegelijkertijd de niet aflatende belangstelling en betrokkenheid van deze collega's hebben mij in mijn wetenschappelijke carrière een goede start gegeven.

Vervolgens werd ik overgeplaatst naar het ITALgebouw waar 'de echte' molekulair biologen zaten. Een hele nieuwe wereld aan onderzoek ging daarvoor mij open. Willem Stiekema werd nu mijn begeleider wat voor ons beide in het begin even wennen was, maar uiteindelijk toch tot het gewenste resultaat heeft geleid. Op het ITAL stond ik op het lab met Jeanne Jacobs die 'hetzelfde' deed als ik, maar dan helemaal anders. Deze tegenstrijdigheid was voor anderen zeer verwarrend, maar wij waren het echter altijd meer met elkaar eens dan de schreeuwpartijen deden vermoeden. Terug op 'De Haaff' kwam ik op het lab de 'transposon jongens' tegen. Onder deze molekulair genetici was het even gewoon om met enzymen als met kruisingschema's te zwaaien. De kritische belangstelling van Mark Aarts en Andy Pereira voor het onderzoek heb ik altijd gewaardeerd evenals de gezelligheid op het lab en in de kroeg.

Als de resistentie toetsen zijn gedaan en de RFLP gegevens binnen zijn, dan is het onderzoek nog niet klaar. Het samenvoegen van beide datasets om de uiteindelijke resultaten te verkrijgen bleek een gevoelige aangelegenheid waar Johan van Ooijen mij enorm bij geholpen heeft. Hij zorgde er tevens voor dat ik niet te wilde conclusies uit mijn onderzoek trok. Ook Piet Stam bleef met een kritisch oog mijn verrichtingen op dit terrein volgen en vooral in de laatste fase van het onderzoek mij van nuttig commentaar voorzien.

Verder zijn er nog vele RFLPcollega's, aaltjesdeskundigen, kamergenoten en meerijders waarmee lunches en andere binnen- en buitenlandse uurtjes werden doorgebracht. Bedankt voor jullie kennis en gezelligheid.

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Nelleke

Curriculum Vitae

Cornelia Maria Kreike werd op 19 juni 1964 in Amsterdam geboren. Na de verhuizing naar Zwanenburg werd het gymnasium bèta doorlopen van 1976 tot 1982 op de scholengemeenschap Sancta Maria in Haarlem. Voor de universitaire studie ging ze weer terug naar Amsterdam om aan de Vrije Universiteit Biologie te studeren van 1982 tot 1987. Alles wat over planten ging had haar belangstelling en zij sloot daarom haar studie af met een hoofdvak Moleculaire Celgenetica en bijvak Bijzondere Plantkunde, de laatste aan de Universiteit van Amsterdam. In 1988 kon zij bij het voormalige SVP, inmiddels opgegaan in het CPRO-DLO, een haalbaarheidsonderzoek van een jaar verrichten naar de toepassing van RFLPs voor de veredeling van mais. Aansluitend hierop is zij begonnen aan dit promotie onderzoek wat in deeltijd (8/10) uitgevoerd is en tot september 1993 geduurd heeft. Het uitwerken van de gegevens en het opschrijven van de resultaten heeft daarna nog een jaar geduurd en uiteindelijk geleid tot deze promotie in juni 1995. In september 1995 hoopt zij als postdoc aan de University of Pennsylvania in Philadelphia te beginnen.