GENETIC AND MOLECULAR ANALYSIS OF THE TOMATO ROOT-KNOT NEMATODE RESISTANCE LOCUS *Mi-1*

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Propositions

1. Genetic analysis is indispensable for positional cloning of genes.

2. The most serious problem associated with chromosome landing remains the resolution that can be achieved with genetic mapping.

3. Structural homology to other resistance genes or complementation of a susceptible phenotype with a candidate resistance gene sequence are not sufficient to claim the isolation of a novel resistance gene.

Dixon *et al.* (1996) *Cell* **84:** 51-459.
Cai *et al.* (1997) *Science* **275:** 832-834.

4. Competition is more stimulating than collaboration.

5. The so-called *avr* genes cause incompatibility rather than loss of virulence, therefore a more accurate term for *avr* genes would be incompatibility genes.

S. Briggs & G. Johal (1994) *Trends in Genetics* **10**: 12-16.

6. The introduction of the Euro as a single European currency should go hand in hand with a European standard for a PhD thesis.

7. Sharing scientific results within a collaboration can be disadvantageous.

8. Public funds for further development of techniques for in vitro fertilization could be better spent on improving facilities for women that combine work and children.

9. Cloning humans equals standstill of evolution.

Propositions from the thesis entitled
"Genetic and molecular analysis of the tomato root-knot nematode resistance locus *Mi-1*"
Tsvetana Liharska, 13 February 1998 Wageningen

To the memory of my grandfather Tsvetko Liharski

Preface

One thing is what you want to do,
another thing is what you can do
and third and fourth is to do it.
Nicolai Haitov, Bulgarian writer

During my five years of research at the Department of Molecular Biology in Wageningen, I have been asked many times if the *Mi* gene has finally been cloned and why does it take us so long to achieve this goal. Here, I must say that I've been fortunate to start working on this project after two PhD students of Pim Zabel, Jacques Aarts and Raymond van Daelen, had done enormous amount of work "towards the isolation of *Mi*".

In August 1992 in a Japanese restaurant in Davis, California a collaboration was set up to join the efforts with two other groups: the group of Valerie Williamson, at the Nematology Department, UCD in California and Keygene in Wageningen. The cloning of *Mi* seemed feasible in one to two years! It was simple as this: Tsveta (MolBi) will take care of the recombinants (Maarten has already made the crosses), Valerie will do the nematode resistance tests and Keygene will provide the (AFLP) markers. No time to waste since the competitors from The States and, later, Germany, were ahead of us. Throughout these years we survived repeated rumours that *Mi* had been cloned, internal discords, and, here they are, jointly and non-jointly written chapters of my thesis.

I am grateful to Pim for all the doors he has opened for me, for his confidence in me and his patience. I will never write scientific English good enough, Pim, but I have learned a great deal from you.

Thanks to my "promotor in shadow" Maarten Koornneef for his guidance in Genetics when I needed it so much. It has always been inspiring working with you, Maarten! I also appreciate the help and the concern of the colleagues from the "vakgroep Erfelijkheidsleer" and from the greenhouse of Henk Kuiper.

I enjoyed very much working with my colleagues from the "tomato group", Ellen, Ruud, Rob, Monique, Guusje, Xiaobo and Jan Hontelez. We surely would have been a even

greater team, Jan, if only we have started earlier working together. I also acknowledge my students Marielle and André for their contribution to the *Mi*-story.

My promotor Ab van Kammen made possible to finish the preparation and the writing of this thesis. I am indebted to him also for his support throughout the years. I will always cherish the time I spent at MolBi. Thanks all my colleagues from the Department 1991-1996 for the pleasant atmosphere!

It has been a real excitement to work with Valerie Williamson ! I am very thankful to the group from Davis for their positive attitude in our collaboration, to Mr. Jan Bezooyen from Department of Nematology for his gratuitous help with the nematode tests, to Taco Jesse and the group from Keygene for my private AFLP course.

Finally, the people who contributed indirectly, but strongly to the realization of this thesis: my family in Bulgaria and my family-in-low Schyns with their understanding and the love; Vesna's friendship and shelter; Philippe, making it look like a "piece of cake".

Contents

Outline of the thesis

The work presented here aims at the molecular isolation of the tomato locus *Mi-1* which confers resistance to root-knot nematodes, a group of wide-spread plant pathogens. Besides of its practical implication, the isolation of disease resistance loci is expected to shed light on the nature of host resistance and to provide material for profound molecular studies of the mechanism of resistance.

The *Mi-1* resistance locus has been introgressed into the cultivated tomato (*Lycopersicon esculentum*) from the wild tomato species *L. peruvianum* and largely exploited in breeding programs during the past fifty years. In *chapter 1*, the properties of *Mi-1*-mediated resistance are described and the availability of additional resistances to root-knot nematodes in *Lycopersicon* species are reviewed. The isolation of the *Mi-1* locus, based on its map position, has been a long standing goal of several laboratories. The state of art of the positional cloning approach to isolate *Mi-1* at the starting time of this work is also presented in *chapter 1*.

To delimit the *Mi-1* locus to a physically small chromosomal segment, it was necessary to obtain a sufficient number of recombinants with known phenotype in this region. Large F_2 populations segregating for *Mi-1* were screened, initially with morphological markers flanking the resistant locus. However, due to suppression of recombination only a few recombinants with crossovers within the *Mi-1* region were found. *Chapter 2* is dedicated to the phenomenon of reduced recombination caused by the presence of introgressions and the proximity to the centromere of the studied region of the tomato genome.

An alternative to recombinant mapping, namely irradiation-induced deletion mapping, was exploited to obtain a high-resolution molecular marker map of the *Mi-1* region (*chapter 3*). Although the obtained deletion mutants were useful for mapping within the recombination silent region of the tomato centromere 6, this approach was found to provide only limited further resolution because of the highly non-random distribution of the deletion breakpoints and the yielding of mainly large terminal deletions.

Additional recombinants for fine mapping of *Mi-1* were identified within other tomato populations, including *Mi-1* segregating populations made between *L. peruvianum* accessions, by screening with flanking PCR-based markers. The molecular analysis of the recombinants

from all crosses with available RFLP and AFLP markers localized *Mi-1* to a region of the genome of less than 65kb and is presented in *chapter 4*.

Next, a spontaneous "loss of function" mutant OT745 that affects both *Mi-1* resistance and resistance to potato aphids (*Meu1* locus) was studied (*chapter 5*). The genetic and molecular data indicated the presence of a mutation, *lmm1*, in the OT745 genotype, that was located within the *Mi-1* introgression and associated with a hypermethylated Cla I site outside the putative *Mi-1* and *Meu1* sequences.

The undertaken genetic approaches to study the *Mi-1* locus are discussed in *chapter 6* in light of the progress made in identifying and characterizing molecularly other disease resistance genes to various pathogens and the recent achievements made in cloning *Mi-1*.

Chapter 1

RESISTANCE TO ROOT-KNOT NEMATODES IN TOMATO

Chromosome landing approach for cloning the Mi-1 locus

Tsvetana B. Liharska and Valerie Moroz Williamson

Host resistance to three major species of root-knot nematodes, Meloidogyne incognita, M. javanica *and* M. arenaria, *has been introgressed in the cultivated tomato (*Lycopersicon esculentum *Mill.) from its wild relative* L. peruvianum. *The trait is dominant and has been located at a single locus,* Mi-1, *on chromosome 6. The* Mi-1*-conferred resistance is associated with a hypersensitive response in nematode-infected tissues; it is not efficient at soil temperatures higher than 28°C, and can be overcome by some "virulent" pathotypes as well as by the species* M. hapla. *Novel resistances, that have broader specificity to root-knot nematodes and are heat-stable, are pursued and identified in the wild tomato germplasm.*

Molecular cloning of the Mi-1 *locus, based on its map position, has been undertaken and reaches its final step. Localizing Mi-1 to a 650kb chromosomal segment, as well as the availability of RAPD and AFLP techniques for isolating molecular markers, has directed a new strategy for positional cloning of* Mi-1 *in recent years, namely chromosomal landing.*

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Introduction

Root-knot nematodes (RKN), genus *Meloidogyne*, belong to the sedentary endoparasites of the family Heteroderidae that are one of the worldwide economically important plant pathogens. The host range of *Meloidogyne* spp. is broad and comprises more than 2000 species from many plant families (Mai, 1985). In few weeks time after the nematode invasion of the roots of a plant, major morphological and physiological changes occur in both organisms. Characteristic for many host plants is the formation of root knots or galls, containing the feeding "giant" cells that provide nutrition for the developing nematode. The outcome for the plant is stunted growth, wilting and susceptibility to other pathogens.

Naturally occurring host resistance against *Meloidogyne* spp. has been found in many crops and related wild germplasm (Sasser *et al.*, 1987). The trait is most often dominant or incompletely dominant and is inherited as a monogenic trait (Fassuliotis, 1987). Although a genetic characteristic, the resistance is conditional and probably depends on the availability and the expression in the nematode of cognate incompatibility factor(s) or avirulence (*Avr*) gene(s). The existence of *Avr* genes has been demonstrated in the cyst nematode-potato system (Janssen *et al.*, 1991), but not yet for the parthenogenetically propagating RKN species. However, the specificity of resistance response as well as the occurrence of resistance-breaking races of RKN support the gene-for-gene mode of action (Flor, 1965) of the RKN resistance genes (Castagnone-Sereno *et al.*, 1996). To elucidate the mechanism of host resistance to RKN it is essential to study the genetic factors mediating this interaction. In recent years there have been major efforts to isolate the gene(s) for resistance to RKN in tomato (*Lycopersicon esculentum* Mill.) (Messeguer *et al.*, 1991; Ho *et al.*, 1992; Ganal and Tanksley, 1996). Tomato is one of the first crops in which RKN resistance has been introgressed by conventional breeding programs and exploited at a large scale (Medina-Filho and Tanksley, 1983). Besides its economical importance, tomato has become a model in plant genetics and offers all the necessary tools to identify genes without knowledge of their product.

In this chapter we describe the availability of RKN resistance in tomato and pay attention to the progress made in the molecular cloning of the first identified and most studied locus, *Mi-1*.

L. peruvianum is the origin of RKN resistance in tomato

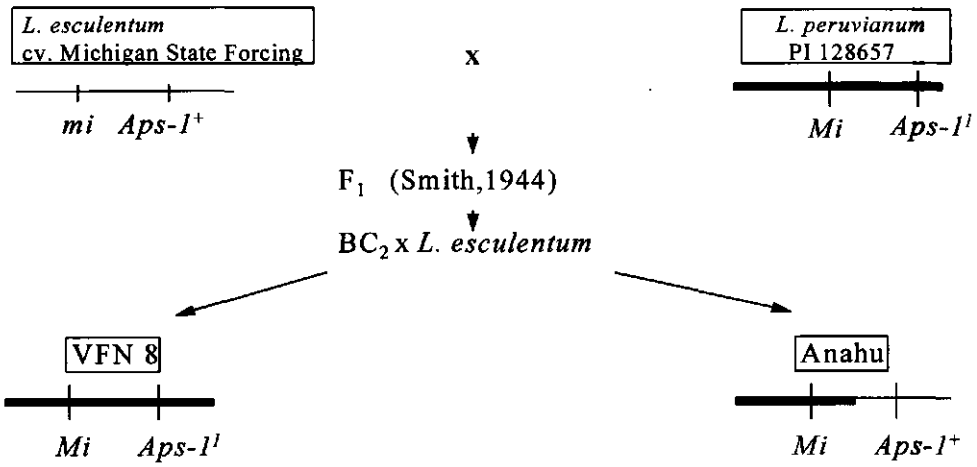

Fig. 1. Breeding of *Mi*-resistance into *L. esculentum*. A single F_1 plant from the cross *L. esculentum* x *L. peruvianum* was backcrossed (BC) to *L. esculentum* as female parent. Progenies from the second backcross were further backcrossed in California and in Hawaii, resulting in two *L. esculentum* lines, VFN 8 and Anahu, respectively. Both lines carry *Mi*, along with an introgression segment from *L. peruvianum* (the black bar) on chromosome 6. The introgression in Anahu line does not include the Aps-1^1 marker.

In the early 1940s screening of large numbers of tomato lines for resistance to RKN revealed that the cultivated tomato is fully susceptible (Bailey, 1941). In the same survey some wild tomato relatives from the genus *Lycopersicon* were included and certain strains of the remote species *L. peruvianum* showed a high level of resistance. Romshe (1942) suggested the use of *L. peruvianum* as a parent for the development of nematode resistant varieties. With the help of embryo culture, a hybrid between *L.esculentum* cv.Michigan State Forcing and *L. peruvianum* accession P.I. 128657 was raised by Smith (1944) (Fig. 1). Backcrossing programs of the single hybrid plant were run simultaneously by two groups, one in Davis, California and the other in Hawaii, and resulted in two *L. esculentum* lines, VFN8 and Anahu, both homozygous for resistance to *M. incognita* (Frazier and Dennett, 1949; Gilbert and McGuire, 1956). The symbol *Mi* was assigned for the resistance in these lines after the first letters of *Meloidogyne incognita* (Gilbert, 1958).

Properties of the *Mi*-mediated resistance

Hypersensitive response

The ineffective stage RKN, J2, are attracted both by *Mi*-resistant and susceptible tomato cultivars. Dean and Struble (1953) studied the mechanism of *Mi*-determined resistance and showed that the nematode invasion of the roots produced an extensive necrotic reaction followed by the disappearance of the J2 from the roots. Whatever is the fate of the nematode, a compatible interaction (formation of giant cells) is not established. Necrosis as a plant defense response is the result of a complex of reactions very similar to those induced by other pathogens, described as hypersensitive response (HR). It is not clear though if the resistance is the result of, or coincides with, or precedes the HR. The first signs of HR can be detected as changes in cell ultrastructure in roots 8 to 12 hours after infection with root-knot nematodes (Paulson and Webster, 1972). Within 2-3 days the HR is localized around the nematode, which either leaves the root or dies there.

Spectrum of resistance

In addition to *M. incognita*, resistance conferred by *Mi* is efficient to the prevalent pathotypes of *M. javanica* and *M. arenaria* (Barham and Winstead, 1957), but not to *M. hapla* (Ammati *et al.*, 1985).

As any host plant resistance, the *Mi* -mediated resistance can be overcome by some races of nematodes. Partial or full susceptibility to *Mi*-breaking populations of *M. incognita*, *M. javanica* and *M. arenaria* are reported to occur naturally or arise under the selection pressure of *Mi*-genotypes in the field or in greenhouse conditions (see Roberts *et al.*, 1990).

Heat instability

At a soil temperature higher than 28°C, *Mi*-containing plants show reduced necrosis and appear susceptible to RKN (Holtzman, 1965). It has been demonstrated by Dropkin (1969b) that only during the first two to three days after penetration of nematodes the temperature determines the course of the interaction in *Mi* plants. This feature of the *Mi*-resistance is not unique. Temperature sensitivity of resistance against root-knot nematodes has been reported for other crops (Cook, 1991) and for other plant-pathogen interactions as well. Considering this, the heat instability of *Mi* resistance could be either an intrinsic feature of the host resistance pathway or a property of the *Mi*-product or /and its interaction with the RKN. Besides, factors that would ensure a heat stable HR of *Mi* may not be present in the *L. esculentum* background neither in the source *L . peruvianum* P.I. 128657 of this resistance

locus (Ammati *et al.*, 1986). Interestingly, at high (32°C) temperature *M. incognita* produce significantly more eggs on susceptible tomato cultivars than at 25°C(Ammati *et al.*, 1986).

Genetics

Gilbert and McGuire (1956) established that the resistance governed by *Mi* is dominant and segregates as a single major locus. The *Mi* locus was mapped on chromosome 6 (Gilbert, 1958). Some controversial data as to the number of genes involved in *Mi*-resistance and their relationship, have been discussed over the years (Sidhu and Webster, 1981). Yet, the history of introgression of *Mi*-resistance in *L. esculentum* (Medina-Filho and Tanksley, 1983) indicates the introgression of a single dominant locus on chromosome 6. Further genetic analysis (Rick and Fobes, 1974) showed a tight linkage between *Mi* and the acid phosphatase-1^1 (*Aps-*1^1) locus. This linkage has been extensively exploited to select indirectly for RKN resistance in tomato breeding programs, excluding lines originating from the Anahu line which has lost the *Aps-*1^1 allele by recombination(Fig.1).

Durability

In the 45 years' history of exploiting the *Mi* resistance in tomato, the resistance has shown remarkable stability. The presence of a *L. peruvianum* segment carrying the *Mi* locus ensures the characteristic resistance described above in any tomato line background. Recombination events breaking the *L. peruvianum* segment with *Mi*, similar to what has happened in the Ana line, have not occurred very often. Also, spontaneous mutations that abolish the function of the *Mi* (Liharska *et al.*, Chapter 5) have been encountered rarely.

Novel genes for resistance to root-knot nematodes are identified in wild tomato germplasm

Until recently, the resistance conferred by the *Mi* locus was the only one identified and utilized in tomato breeding. The need to broaden the genetic basis of resistance prompted new attempts to screen wild tomato species for resistance to RKN (Ammati *et al.*, 1985; Lobo *et al.*, 1988; Roberts *et al.*, 1990; Cap *et al.*, 1991; Scott *et al.*, 1991; Yaghoobi *et al.*, 1996; Veremis and Roberts, 1996a,b,c). Resistance effective at high temperatures and against *M. hapla* as well as against *Mi*-breaking pathotypes, was pursued. From all the *Lycopersicon* species tested, only some accessions from the *L. peruvianum* complex, different from the original *Mi* source, were found to possess a variety of new resistances to RKN (summary in

Table 1. Identified resistances to root-knot nematodes in "*L. peruvianum* complex".

Accessions	Resistance specificity[1]	temperature limit	Genetics locus	map position	references
L. peruvianum P.I.128657	*M. incognita*, *M. arenaria*, *M javanica*	28°C	***Mi-1***	chr.6	Bailey, 1941 Medina-Filho and Tanksley, 1983
P.I.270435 -clone 2R2	*M. incognita*	28°C	***Mi-1***	chr.6	Cap *et al.*, 1993
	M. incognita	heat stable	***Mi-2***	nd	
	M.i. 557R[2]	28°C	***Mi-8***	nd	Yaghoobi *et al.* 1995
	M. javanica	28°C	-	nd	Veremis and Roberts,
	M. javanica	heat stable	-	nd	1996b,c
P.I.270435-clone 3MH	*M. incognita*	heat stable	***Mi-6***	nd	Cap *et al.* , 1993
	M.i. 557R	28°C	***Mi-7***	nd	Roberts *et al.* 1990
	M. javanica	28°C	-	nd	Veremis and Roberts, 1996b,c
P.I. 129152	*M. incognita*	heat stable	-	nd	Ammati *et al.*, 1985,
LA 1708-I	*M. incognita*	heat stable	***Mi-4***	nd	Veremis and Roberts, 1996a
P.I.126443-clone 1MH	*M.i.* 557R	28°C	***Mi-3***	chr.12	Cap *et al.*, 1991, 1993
	M. incognita, *M. javanica*	heat stable	***Mi-5***	chr.12	Yaghoobi *et al.*, 1995
	M. javanica	28°C	-	nd	Veremis and Roberts,
	M. javanica	heat stable	-	nd	1996b,c
	M. hapla	heat stable	-	nd	Ammati *et al.*, 1985, 1986
L.chilense LA2884	*M. javanica*	heat stable	-	nd	Veremis and Roberts, 1996c

Notes: 1) resistance to species or pathotypes of RKN that has been tested and confirmed; 2) *M. incognita* virulent strain 557; nd) not determined

Table 1). Backcrossing to a susceptible *L. esculentum* cultivar revealed the dominant nature of the newly identified traits. Test for allelism with *Mi* or linkage with the *Aps-1*^{1} locus were exploited to classify the new loci and to discriminate between them and *Mi*. The genetic analysis of these resistances and their properties point out that they are determined by loci distinct from *Mi* (Cap *et al.*, 1993; Yaghoobi *et al.*, 1996; Veremis and Roberts, 1996a). To some, symbols from *Mi-2* to *Mi-8*, have been assigned, and the original *Mi* locus is referred to as *Mi-1*. The relationship among the novel resistances needs to be studied further as some might appear to be allelic. To date, resistance to a virulent isolate of *M. incognita* (557R) has been mapped as a single locus *Mi-3* on the telomeric end of chromosome 12 in tight linkage to the heat-stable resistance to *M. incognita* (*Mi-5)* (Yaghoobi *et al.*, 1995; Veremis and Roberts, 1996a). The mechanisms by which the novel genetic factors provide resistance against RKN have not been studied. It is not clear yet if they are associated with HR like the *Mi-1* conferred resistance.

The efforts to introduce the newly identified resistances from *L. peruvianum* into *L. esculentum* have been hampered by the remoteness of the two species and the lack of cross-compatibility. Rescuing the interspecific hybrids by embryo cultures has been successful for some, but not all *L. peruvianum* genotypes (Smith, 1944; Cap *et al.*, 1991; Scott *et al.*, 1991). Another approach that utilizes bridging lines, made between some "easy" crossable *L. peruvianum* genotypes and *L. esculentum,* has also not been successful so far (Veremis and Roberts, 1996a).

Molecular genetics of the *Mi-1* locus

Molecular studies of *Mi-1* were undertaken in the 1980's and were aimed at both isolation of DNA markers for indirect selection for resistance and cloning the sequence encoding the resistance based on its map position.

As a first step of a map-based strategy for cloning *Mi-1*, closely linked markers were identified as possible starting points for chromosome walking (Klein-Lankhorst *et al.* 1991; Messeguer *et al.* 1991). This included the major work of cloning of the *Aps-1*^{1} allele and converting it to a DNA marker (Aarts *et al.* 1991; Williamson and Colwell 1991). The map position of *Mi-1* on chromosome 6 was refined, using a number of genetic crosses (Weide *et al.* 1993) and the distance between the flanking markers GP79 and *Aps-1* was estimated to be less than 2cM (Messeguer *et al.* 1991; Ho *et al.* 1992). This distance in tomato can be translated to approximately 1,5 Mb (Tanksley *et al.* 1992) and indeed, long-range physical mapping of the two markers, GP79 and *Aps-1*, revealed that they are at least 1,2Mb apart (van

Daelen *et al.* 1993). Chromosome walking on such a large distance was not feasible, in spite of the availability of YAC libraries of nematode resistance genotypes (Martin *et al.* 1992; van Daelen 1995). The expected presence of repetitive sequences in this region, due to its proximity to the centromeric heterochromatin, seemed to be a major obstacle for conventional chromosome walking. Indeed, during the last years deletion and cytogenetic mapping have positioned the *Mi-1* locus and the GP79 marker to the short arm of the chromosome (Fig. 2) near the border with the heterochromatin (van Wordragen *et al.* 1994; Xiaobo Zhong, in preparation) and separated it from the *Aps-1* marker by the centromere.

Screening of nematode resistant tomato varieties with the available RFLP markers has resulted in the identification of one introgression line, Motelle, which is nematode resistant and has none of the *L. peruvianum* alleles for GP79 and *Aps-1* (Messeguer *et al.*1991; Ho *et al.* 1992). The extent of the *L. peruvianum* introgression carrying *Mi-1* in Motelle has been estimated at approximately 650kb (P.Vos, personal communication).

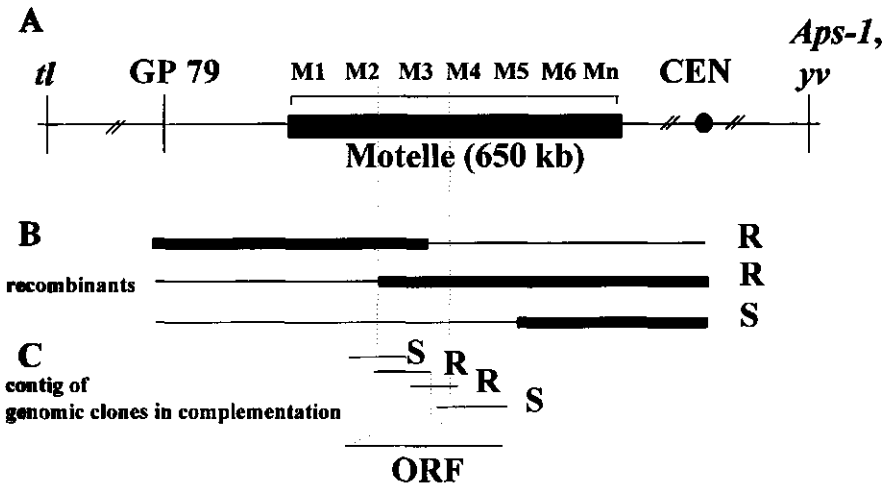

Fig. 2. Chromosome landing approach for cloning the *Mi-1* locus. **A.** The line Motelle has approx. 650 kb of *L. peruvianum* introgression (the black bar), carrying the *Mi-1* locus on the short arm of chromosome 6. A considerable number of molecular markers (M1 - Mn) randomly distributed within these 650 kb are needed. **B.** Recombinants from crosses between resistant (the black bar) and susceptible(the line) genotypes with crossover within the Motelle region and with known phenotype (R or S). Molecular marker mapping of the crossover points reveals molecular markers (M2 and M3) in the vicinity of *Mi -1*. **C.** Complementation of a susceptible tomato genotype with selected genomic clones between the markers M2 and M3 . The phenotype of the transformants will further indicate the genuine coding sequence(s) for *Mi -1* .

Localizing *Mi-1* to this small chromosomal segment, as well as the availability of RAPD and AFLP techniques for isolating additional molecular markers, has directed a new strategy for positional cloning of *Mi-1* in recent years, namely chromosomal landing (reviewed by Tanksley *et al.* 1994) (Fig. 2). The approach is widely applicable and aims at tagging the gene of interest by employing large numbers of molecular markers (Fig. 2 A) and recombinants with known phenotype (Fig. 2 B). Complementation of a susceptible or mutant genotype is required to indicate the genomic clone containing the true gene.

Acknowledgments

We thank John Veremis for providing in-press manuscripts and Ab van Kammen and Maarten Koornneef for critical reading the manuscript.

Chapter 2

TOMATO CHROMOSOME 6 : EFFECT OF ALIEN CHROMOSOMAL SEGMENTS ON RECOMBINANT FREQUENCIES

Tsvetana B. Liharska, Maarten Koornneef, Monique van Wordragen, Ab van Kammen, and Pim Zabel

Variation in recombinant frequencies at two adjacent intervals on chromosome 6 of tomato (Lycopersicon esculentum *Mill.) has been studied in seven lines that differ in the amount and origin of introgressed segments from wild species. These lines were all crossed to a genotype homozygous recessive for the markers* tl, yv *and* c *defining the centromere spanning region* tl-yv *and long arm region* yv-c, *respectively. Recombinants were identified in large* F_2 *populations consisting of over 30,000 plants in total. Application of molecular markers provided additional information on the distribution of crossover events within the centromere-containing interval* tl-yv. *A decrease in recombination at the marked intervals correlated with the presence of an alien segment. Suppression of recombination was up to six-fold in the centromere spanning interval* tl -yv *depending on the source and the size of the introgression, and was restricted to the alien segments with no strong effect on the neighboring intervals.*

This chapter has been published in *Genome* (1996) **39:** 485 -491.

Introduction

In plants, recombinant frequencies for pairs of genetic markers have often been observed to vary, depending on the crosses involved. Differences have been reported between female and male meiosis for the frequency and, in some cases, the distribution of crossover events in maize (Robertson 1984), *Pinus* (Moran *et al.* 1983; Groover *et al.* 1995), *Arabidopsis* (Vizir and Korol 1990), *Lycopersicon* (de Vicente and Tanksley 1991; van Ooijen *et al.* 1994) and *Brassica* (Lagercrantz and Lydiate 1995). Besides a genetic basis underlying variation in recombination, environmental factors such as temperature (Gavrilenko 1984) and passage through *in vitro* culture (Compton and Veilleux 1991), appear to play a role in affecting the rate of meiotic recombination as shown for tomato.

Large variations in intergenic recombination among different genotypes of petunia have been attributed to the activity of a nuclear gene (*Rm-1*) modulating the recombination across the genome (Cornu et *al.* 1989). On the other hand, there is also substantial evidence showing a direct relation between recombinational variation and the chromosomal structure. For instance, large deletions in the short arm of chromosome 6 in petunia correlated with increased recombination in a marked region of the long arm of the same chromosome (Gerats *et al.* 1984). More examples of the relationship between chromosomal structure and recombinational variation come from studies dealing with introgressions of wild germplasm into cultivated species. In backcrosses of introgression lines of tomato (Rick 1969; Paterson *et al.* 1988; Paterson *et al.* 1990; Messeguer *et al.* 1991) and barley (Görg *et al.* 1993) to their respective recurrent parents, a several fold suppression of recombination was often associated with the alien segment. In those cases, structural differences between the genomes of the donor and the recurrent parent could account for the reduced level of recombination (Messeguer *et al.* 1992; Ho *et al.* 1993).

Although there is an increasing amount of data showing reduction in recombinant frequency due to introgression, no systematic studies of this phenomenon have been conducted to date. The cultivated tomato (*L. esculentum* Mill.) provides a suitable system for such an analysis (Hille *et al.* 1989; Rick 1991) as both detailed classical and molecular linkage maps of its entire genome have been constructed (Tanksley et al. 1992), as well as integrated maps of chromosome 1 (Balint-Kurti *et al.* 1995), 3 (Koornneef *et al.* 1994; van der Biezen *et al.* 1994) and 6 (Weide *et al.* 1993; Van Wordragen *et al.* 1994; Van Wordragen *et al.* 1996) . Moreover, the seven wild relatives of tomato have been used for decades as genetic donors of valuable traits in breeding programs, thus providing a large reservoir of introgression lines.

Here, we compare for seven tomato genotypes that differ in the size and origin of an introgressed segment on chromosome 6 the recombinant frequencies at two adjacent intervals spanning the centromere (*tl- yv*) and a portion of the long arm (*yv -c*) (Khush and Rick 1968), respectively, using the introgression-free line Moneymaker as control. All lines were crossed to the male parent *L. esculentum* line W607 carrying the morphological markers *tl (thiamineless), yv (yellow virescent)* and *c* (potato leaf) and over 30,000 F_2 plants were scored for segregation of the morphological markers. The distribution of recombination within the centromere-spanning interval *tl-yv* was further established using molecular markers. The suppressive effect of the introgressions on the frequency and distribution of meiotic recombination within the marked intervals is discussed.

Materials & methods

Plant material and the construction of the marker line W607

The genetic composition of chromosome 6 for the lines used as female parents in the crosses to W607 has been determined previously by molecular markers (Messeguer et al. 1991; Ho et al. 1992; Weide et al. 1993; Van Wordragen et al. 1994) and is shown in Fig. 1B. All the lines that contain an *L. peruvianum* introgression are derived from the same hybrid of the *L. esculentum x L. peruvianum* PI 128657 cross made by Smith (1944).

The male parent, W607, was derived from a cross of *L. esculentum* lines LA758 and LA1189. LA758 originates from a spontaneous *thiamineless* mutation (Langridge and Brock 1960) in the cultivar Anahu that carries an *L. peruvianum* introgression on the short arm of chromosome 6 (Ho et al. 1992). LA1189 is homozygous for the *yv* and *c* mutations. Because no plant was found among the few hundred F_2 progeny of LA758 x LA1189 that was double recessive for *tl, yv* and *c* , ten F_2 plants that showed the yv, c phenotype were subsequently self pollinated. Among the offspring of these plants, individuals homozygous for *tl, yv* and *c* were found and they are referred to as marker line W607. Molecular analysis of W607 revealed a small *L. peruvianum* segment in the close vicinity of the *tl* locus (Fig. 1B).

The parents of all crosses, the hybrids and their selfed progeny were grown in greenhouses in Wageningen in the period 1992-1994 with additional light and heating during the winter.

All tomato genotypes used in this work are listed, along with their sources, in Table 1.

F_2 analysis

Phenotypic classification , which allowed the identification of all informative recombinant classes (*tl* yv^+/ *tl yv*, tl^+yv /*tl yv*, *yv* c^+/*yv c*, yv^+c /*yv c*,), was performed in the F_2 populations at the seedling stage. At two to three weeks after sowing, the *thiamineless* phenotype could be recognized and the plants were rescued by weekly application of thiamin to the soil (~0.1mg per pot). At this stage, the *c* character was also scored. The tl^+yv / *tl yv* (*c* or c^+) recombinants were selected among the slowly growing *yellow virescent* plants within the next few weeks.

Estimates of recombinant frequencies and their standard errors were calculated with the computer program RECF2 that calculates recombinant fractions from pairwise segregation data and has been designed to account for certation when recessive deficits are observed (Koornneef and Stam 1992). Differences in the recombinant values for the marker pairs between the different crosses were examined by using the χ 2 test.

Table 1

Line	Source	Reference
Motelle	Laterrot	Laterrot (1987)
VFNT Cherry	TGC-LA1221 *a	Young et al. (1989)
83M/R	De Ruiter Seeds	Klein-Lankhorst et al. (1991)
Ontario 7620	INRA, France	Kerr et al. (1980)
Vetomold	CPRO-DLO *b	Langford (1937)
WSL-6	WAU *c	Weide et al. (1993)
Moneymaker	Nunhem Seeds	
LA758	TGC	Langridge and Brock (1960)
LA1189	TGC	Rick (1988)
W607	See text	This work

*a Tomato Genetics Cooperative Seed Stock Center, Davis.

*b Center for Plant Breeding and Reproduction Research - DO, Wageningen

*c Wageningen Agricultural University.

DNA isolation and molecular analysis

Plant nuclear DNA for Southern analysis was isolated from leaves as previously described (van der Beek et al. 1992). PCR analysis was carried out on DNA samples prepared as described by Wang and Cutler (1993). Labelling of RFLP marker GP79 and Southern blot hybridisation were carried out as described elsewhere (Klein-Lankhorst et al. 1991). SCAR marker REX1, which maps within the *tl-yv* interval and very close to the nematode resistance gene *Mi*, was analyzed following the protocol of Williamson et al. (1994).

Results

Variation of recombination frequencies among lines with introgressions from different donor species

Seven *L. esculentum* lines differing in the size and origin of an introgression on chromosome 6 (Fig. 1B) were crossed as female parent to the triple recessive marker line W607 and in total 30,967 F_2 progeny plants were scored at the seedling stage for the phenotypes *thiamineless*, *yellow virescent* and potato leaf. The calculated recombinant values for the *tl-yv* and *yv-c* intervals are presented in Table 2.

In each of the three lines, VFNT Cherry, WSL6 and Vetomold, differing both in the origin and the amount of alien sequences present on chromosome 6 (Fig. 1B), the

introgression spans the interval *tl-yv* and, thereby, carries a centromere 6 (*CEN 6*) alien to *L. esculentum.* The mean recombinant frequency for the *tl -yv* pair was different for each line and, in all cases, less than the frequency (6.6%) found with the introgression-free Moneymaker line.

Table 2. Frequency of recombination between *tl, yv* and *c* in crosses with line W607.

			tl-yv interval		*yv-c* interval	
Female parent	Source of introgression	Total F 2	No. of recombinants	Recombinant percentage	No. of recombinants	Recombinant percentage
Moneymaker	none	4197	248	6.6 ± 0.4^{a}	1381	44.5 ± 1.1^{a}
Vetomold	*L.pimpinellifolium*	3570	188	5.5 ± 0.4^{a}	1203	44.3 ± 1.2^{a}
WSL6	*L.pennellii*	2311	43	1.9 ± 0.3^{b}	480	25.0 ± 1.1^{b}
VFNT Cherry	*L.peruvianum*	7502	66	1.0 ± 0.1^{c}	1784	45.6 ± 1.3^{a}
83M/R	*L.peruvianum*	2019	39	2.0 ± 0.3^{b}	682	44.4 ± 1.6^{a}
Motelle	*L.peruvianum*	6720	208	3.3 ± 0.2^{d}	1689	42.3 ± 0.8^{a}
Ontario-7620	*L.peruvianum* , *L.pimpinellifolium*	4648	176	3.9 ± 0.4^{d}	1600	44.8 ± 1.0^{a}

Note: Source of introgressions on chromosome 6. Values followed by different letters differ significantly in pairwise χ^2 analysis of the recombinant and non-recombinant classes ($P<0.05$).

The large *L. peruvianum* chromosome segment in VFNT Cherry exhibited the most suppressive effect on recombination (approx. 7-fold) within the *tl-yv* interval. An introgression of about the same size but originating from *L. pimpinellifolium* in the line Vetomold led only to a small and non-significant reduction in recombinant frequency. Unlike the centromere interval *tl-yv* , the genetic distance *yv-c* in crosses involving VFNT Cherry and Vetomold did not differ significantly from the control Moneymaker.

Line WSL6, in which a major part of chromosome 6, including the marked intervals, has been replaced by the *L. pennellii* counterpart (Weide *et al.* 1993), showed for both the *tl-yv* and *yv-c* intervals a lower recombination rate as compared to the control Moneymaker. The reduction in recombinant frequency was more severe in the centromere-containing interval *tl-yv* (approx. 3.5-fold) than in the paracentric *yv-c* interval (approx. 2-fold). Recombination rates for both intervals in the control cross Moneymaker were very close to the ones from the classical map of chromosome 6 established previously (Weide *et al.* 1993) (Fig. 1A).

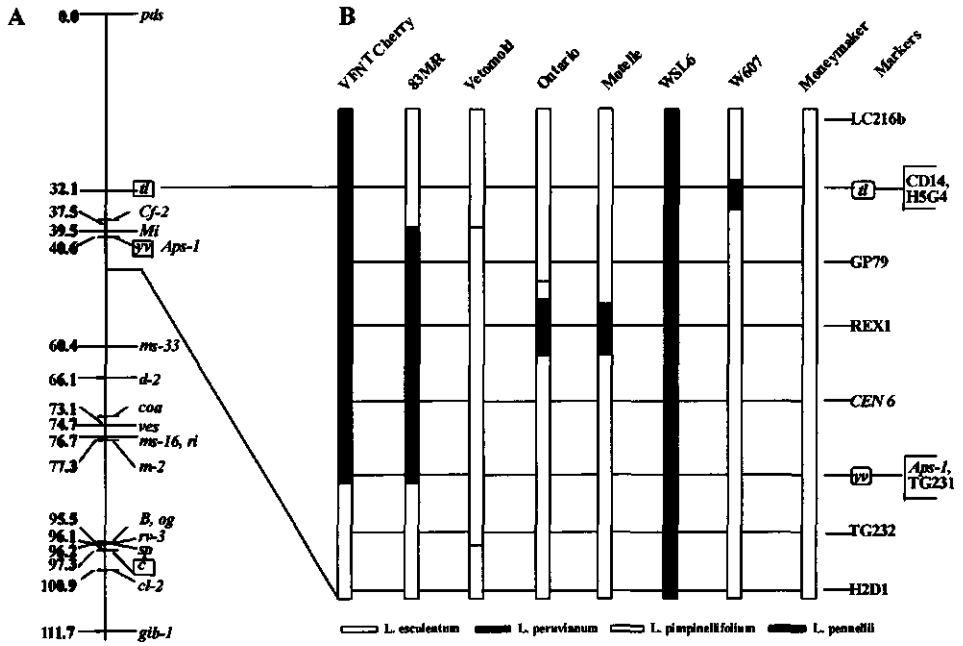

Fig. 1 A. Classical linkage map of tomato chromosome 6 (Weide et al. 1993). B. Map of the *tl-yv* region showing the relative order of the classical and molecular markers around the centromere (Messeguer et al. 1991, Ho et al. 1992; van Wordragen et al. 1994) and the composition of the tomato lines, used in this study. Introgressions from *Lycopersicon* species are depicted by bars and their sizes are not on genetic or physical scale.

Effect of the size of an L. peruvianum introgression on the recombination in the tl-yv interval

Comparison of the recombinant frequencies within the *tl-yv* interval in four of the lines that carry different amounts of an *L. peruvianum* introgression (Fig. 1B) revealed a negative correlation between the extent of introgression and the rate of recombination. Among these lines, VFNT Cherry hybrids showed the most severe suppression of recombination relative to the control Moneymaker. Remarkably, however, even the small *L. peruvianum* segment that is present in Motelle and Ontario and only amounts to 750 kb and 900 kb respectively, (P.Vos, Keygene, personal communication) significantly reduced the recombinant frequency in the *tl-yv* interval (approx. 2-fold) (Table 2, Fig. 2). As compared to Motelle/Ontario and VFNT Cherry, line 83M/R carries an introgressed segment of intermediate size and as such showed an intermediate level of recombination (2%) in the *tl-yv*

interval. In conclusion, these data indicate that the frequency of recombination decreased with the extent of the alien chromosomal segment.

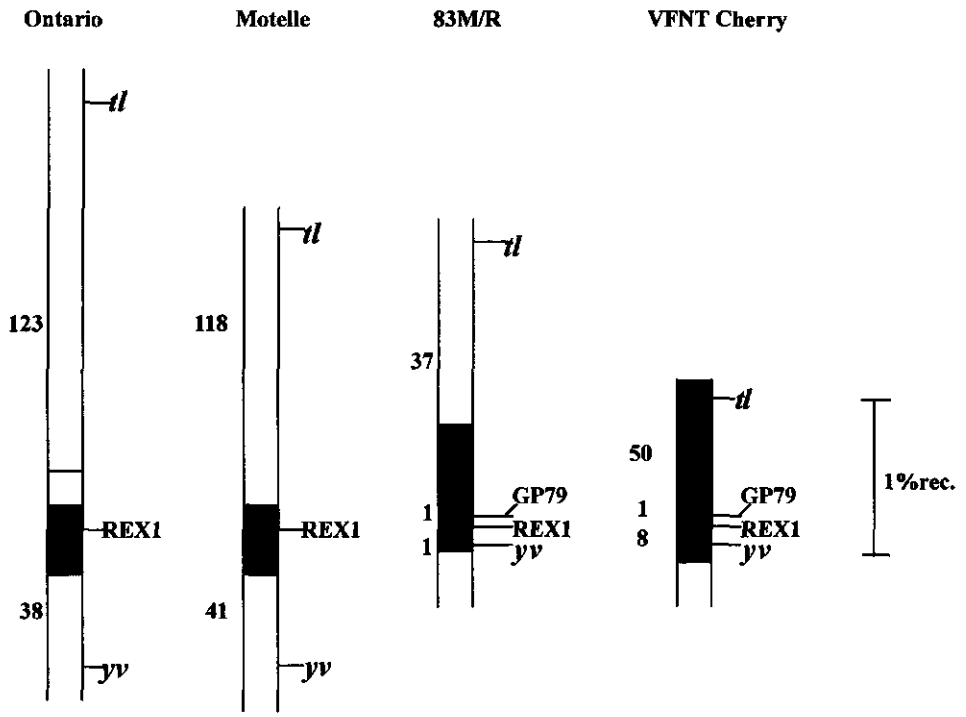

Fig. 2. Frequencies of recombination between *tl* and *yv* and the molecular markers estimated in crosses W607 with lines that differ in the extent of *L. peruvianum* introgression (the black bar). Recombination decreased with the size of the introgression. Numbers indicate the number of recombinants that were identified for these intervals when a subset of the *tl-yv* recombinants were analysed for REX1 and GP79.

Distribution of crossover events across the tl-yv interval

As for the *tl-yv* interval, nearly 500 recombinant plants were obtained from the crosses with VFNT Cherry, 83M/R, Motelle and Ontario. Subsequent application of the PCR marker REX1 (Williamson *et al.* 1994) that maps within this interval (Van Wordragen *et al.* 1994) and resides on the smallest introgressed *L. peruvianum* region present in Motelle (Fig. 1) enabled us to precisely assess the relative distribution of recombination events across the *tl-*

Recombinant frequencies of the *tl*-REX1 and REX1-*yv* pairs in the Ontario cross were almost the same as in the Motelle cross, indicating that the additional small *L. pimpinellifolium* segment present in Ontario did not affect crossing over (Fig. 2). Although the overall reduction of recombination at the *tl-yv* interval in 83M/R was stronger than in Ontario and Motelle, the *tl*-REX1 recombinant frequency in 83M/R was similar to those in Ontario and Motelle (2%-2.5%). Thus, severe suppression of recombination within the *tl-yv* interval in 83M/R was largely confined to the REX1-*yv* subinterval, that is occupied completely with *L. peruvianum* sequences and carries the *CEN 6*. The interval *tl*-REX1 in VFNT Cherry showed twice as less (0.9%) recombination. These data clearly show that reduction of recombination occurs only in the interval carrying the introgression and does not extend into adjacent intervals.

A Chi-square test with a correction for continuity was applied to compare the distribution of *tl-yv* recombinants over the intervals *tl*-REX1 and REX1-*yv*. This analysis showed that the difference in relative distribution between VFNT Cherry and 83M/R is not significant ($P>0.05$) as is the difference between the Ontario and Motelle cross. Neither was the difference between VFNT Cherry and both, Ontario and Motelle significant. However, the distribution of crossover events in 83M/R differs significantly from that in both Ontario and Motelle. Adding the data for VFNT Cherry and 83M/R together and comparing these with the combined data for Ontario and Motelle indicates a significant ($P<0.01$) difference in recombination pattern on both sides of the REX1 marker when the heterozygotes differ in the presence of *L.peruvianum* sequences around the centromere.

Thus, not only the amount but also the pattern of distribution of recombination is affected by the presence of alien chromosomal sequences (Fig. 2).

Discussion

Our results show heterogeneity for recombinant frequencies among seven tomato genotypes at two adjacent intervals, *tl-yv* and *yv-c*, spanning a large region of chromosome 6. Selection of recombinants with the easily scorable morphological markers *tl* , *yv* and *c* allowed the analysis of large F_2 populations and this, together with the uniform external conditions for all the crosses, reduced the effect of factors other than the genetic background on meiotic recombinant frequency.

Decreased recombination was observed when alien chromosomal sequences were present at the marked intervals. The conclusion that introgressions, present in heterozygous

state, directed the local decrease in recombinant frequencies is supported by the correlation between the amount of decrease and the extent and origin of the alien segment. Remarkably, even a *L. peruvianum* segment as small as 650 kb is sufficient to cause a two fold reduction in recombinant frequency in the physically much larger *tl-yv* interval. The larger is the alien segment, as illustrated by the 83M/R and VFNT Cherry lines, the more pronounced is the suppressive effect. There are related examples in yeast showing that in a marked interval of 9kb the introduction of sequence divergence as small as nine RFLP's in heterozygous state reduces the frequency of crossing-over approximately two-fold (Borts and Haber 1987).

Reduction of recombination occurs in relation to the origin of introgression

The suppressive effect of introgressions originating from different sources on the recombinant frequency in the *tl-yv* interval ranged from non-significant for a segment derived from *L. pimpinellifolium* in Vetomold to more than 6-fold for the *L. peruvianum* derived segment in VFNT Cherry. Apparently, the sequence divergence between the regions involved in chromosome pairing plays a major role in directing the efficiency of recombination. Indeed, RFLP analysis of phylogenetic relationships and genetic variation in the genus *Lycopersicon* (Miller and Tanksley 1990) places *L. pimpinellifolium* closest to the cultivated tomato and *L. pennellii* and *L. peruvianum* as most divergent. As for the whole genome, we often found little or no polymorphism at the *tl-yv* region between *L. pimpinellifolium* and *L. esculentum* , whereas *L. pennellii* and *L. peruvianum* were polymorphic in that interval for any sequence examined (Liharska, Van Wordragen, unpublished). The divergence between *L. peruvianum* and *L. esculentum* in this region is so great that some sequences isolated from *L. peruvianum* do not have a homologue in domestic tomato (Ho *et al.* 1993). Dixon *et al.* (1995) observed a genetic distance between *tl* and *yv* of 3.54 cM when they analyzed the progeny of the cross of Vetomold with a *tl* mutant carrying a much larger *L. peruvianum* introgression than W607. The suppression of recombination that we observed in *L. peruvianum / L. esculentum* heterozygotes apparently is also present in *L. pimpenellifolium / L. peruvianum* heterozygotes, which agrees with the remote position of *L. peruvianum* relative to the other two species.

Other authors (Balint-Kurti *et al.* 1994) have found a similar relationship between the level of recombination and the relatedness of the alien segment in another marked region of the *L. esculentum* genome. There, an introgression on tomato chromosome 1 originating from the remote species *L. hirsutum* had a more suppressive effect on recombination than an *L. pimpinellifolium* segment .

Stronger suppression of recombination in the centromere interval

When both the *tl-yv* and *yv-c* intervals carried *L. pennellii* homologues, the reduction in recombination was larger in the centromere interval *tl-yv* than in the paracentric *yv-c* interval. The mere difference in lengths between these two intervals can not account for the observed differential suppression of recombination since a similar result was obtained with the VFNT Cherry cross. In VFNT Cherry the presence of *L. peruvianum* sequences resulted in seven-fold reduction of recombinant frequency in the centromere-spanning REX1-*yv* interval whereas in the *tl*-REX1 interval the reduction was only two-fold, compared with Ontario and Motelle that contain *L. esculentum CEN 6* (Fig. 2).

A considerably higher reduction of recombination in the centromere-containing interval as compared to paracentric intervals has also been reported by Rick (1969) for *L. pennellii* introgressions in tomato chromosomes 6, 8 and 11. Some features of the centromere region may account for the observed effect. First, as indicated by the clusters of markers on the linkage map (Tanksley *et al.* 1992) and by the distribution of the recombination nodules along the tomato chromosomes (Sherman and Stack, 1995) the centromere regions of tomato exhibit less recombination than other parts of the genome. This genome specific pattern of distribution of recombinant frequencies along the chromosomes is a common feature of eukaryote genomes and it is said to be under complex fine control of recombination (Simchen and Stamberg, 1969). Another feature of the centromere region that may also explain our results and can add to the first is that the centromere and the linked heterochromatin mainly consist of non-coding sequences that evolve faster and, consequently, will be more divergent among the species than the rest of the genome.

Recombination reduction within the introgression does not have a strong effect on the neighboring interval

As the recombinant frequency of the interval *yv-c* was not affected in lines with introgressions within *tl-yv* (Vetomold, VFNT Cherry, 83M/R, Ontario and Motelle), suppression of recombination at the *tl-yv* interval is apparently not due to an overall lower rate of crossing over for the whole chromosome 6, nor the result of an relative enhancement of the recombination rate in the introgression-free *yv-c* region. Such a compensation of the lower level of recombination in introgressed regions might occur if regions of chromosome 6 more distal than the examined *yv-c* interval are involved as suggested by Rick (1969). Another possibility is that the centromere acts as a barrier to such compensation at *yv-c* on the long arm, though such a role of the *CEN 6* is not supported by our results obtained with the 83M/R line, where the severe recombination reduction in the REX1- *yv* region did not have

significant effect on recombination in the adjacent *tl*-REX1 interval on the short arm of chromosome 6.

Acknowledgments

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Chapter 3

MOLECULAR MAPPING AROUND THE CENTROMERE OF TOMATO CHROMOSOME 6 USING IRRADIATION-INDUCED DELETIONS

Tsvetana B. Liharska, Jan Hontelez, Ab van Kammen, Pim Zabel and Maarten Koornneef

*Irradiation-induced deletion mapping was exploited to construct a detailed locus order map around the centromere of tomato chromosome 6 (*CEN 6*). An F1 hybrid heterozygous for the marker loci thiamineless (*tl*), yellow virescent (*yv*) and potato leaf (*c*), and homozygous recessive for the nematode resistance gene mi was pollinated with γ-irradiated pollen from cultivar VFNT Cherry carrying the wild type alleles at the corresponding loci. A dose of 100 Gy was found optimal for inducing mutants. By screening for pseudo-dominant plants showing the marker phenotypes and/or nematode susceptibility, 30 deletions encompassing one or more of the four loci were detected in the M_1 generation. Molecular marker analysis revealed that 29 of these mutants included the tl and mi loci on the short arm and originated from terminal deletions of different sizes. Remarkably, the breakpoints of these deletions were not randomly distributed along the short arm but located within the centromeric heterochromatin. Only one yv interstitial deletion and no c mutations on the long arm of the chromosome were detected. Mapping of the various chromosomal breakpoints in the isolated mutants permitted the resolution of a cluster of molecular markers from the centromeric heterochromatin that was hitherto unresolvable by genetic linkage analysis. The usefulness of such a deletion mapping approach for whole-genome mapping is discussed.*

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Introduction

As with most centromeric regions, there is little recombination close to the centromeres of the tomato (Tanksley *et al.* 1992). Consequently, to resolve the order of loci in such regions, very large segregating populations must be screened. Morphological markers closely flanking the region of interest can be helpful in pre-selecting for crossover events within the marker interval thereby rendering the number of plants for molecular analysis manageable (Koornneef *et al.* 1994; Van Wordragen *et al.* 1994; Dixon *et al.* 1995; Liharska *et al.* 1996).

In resolving the order of loci that can not be separated by genetic linkage analysis, mapping the breakpoints of overlapping radiation-induced mutations by means of molecular markers provides an alternative that has been widely applied to the genome of *Drosophila*, mouse and human. Similarly, in higher plants, irradiation-induced mutations have been utilized for many years in classical genetic studies (Khush and Rick 1968; McClintock 1984) and more recently to provide starting material for the isolation of genes (Sun *et al.* 1992; Okubara *et al.* 1994; Anderson *et al.* 1996). Ionizing radiation is highly energetic and causes chromosome breaks leading to deletions (as well as other types of chromosomal rearrangements) that are not correlated with the recombination frequency in a particular region and, hence, allow mapping of markers that remain difficult to resolve by genetic linkage analysis. On the other hand, deletion breakpoints may not be distributed randomly along the chromosome, as shown by Khush and Rick (1968). The availability at present of a rich source of molecular markers allows a much more detailed analysis of the distribution of breakpoints than was feasible in the days of Khush and Rick.

In the present study, we have investigated the potential of γ-irradiation to resolve the order of molecular markers around the centromere of chromosome 6 (*CEN 6*) using a combined genetic and morphological approach. This region is of great interest because it contains important disease resistance genes such as *Mi-1*, *Cf-2* and *Cf-5*, *Meu-1* and the virus tolerance gene *Ty-1* (Messeguer *et al.* 1991; Ho *et al.* 1992; Dickinson *et al.* 1993; Kaloshian *et al.* 1995; Zamir *et al.* 1994). Our previous attempts to construct a high-resolution molecular linkage map of this region, which is flanked by the morphological markers *tl* (*thiamineless*) and *yv* (*yellow virescent*), have been hampered by the very low recombination frequency within this specific interval (Liharska *et al.* 1996). Cytogenetic analysis of *tl* and *yv* pseudo-dominants mutants by Khush and Rick (1968) showed that these two loci reside on different sides of the centromere. Here we show that deletion mapping provides an effective alternative to ordering markers in an otherwise, silent in recombination region of the chromosome.

Materials & methods

Plant material

The female parent (*Tl/tl, Yv/yv, C/c, mi/mi*) used in crosses with irradiated pollen was a hybrid between W607 and the cultivar Moneymaker. The homozygous marker line W607 (*tl/tl, yv/yv, c/c, mi/mi*) could not be used for the present experiments because it is a poorly growing plant with a low seed setting (Liharska *et al.* 1996). Tomato line VFNT Cherry (LA1221) carrying dominant alleles at the tester loci *tl, yv , c* and *mi* was used as a pollen donor. This line carries an *L. peruvianum* introgression covering the short arm, CEN 6 and the centromere proximal part of the long arm (Fig. 2; Messeguer *et al.* 1991; Ho *et al.* 1992) . The parents and the offspring were grown in a greenhouse in Wageningen during the years 1994 and 1995.

Mutagenesis

Using an "electric bee", mature pollen from VFNT Cherry plants was collected in a glass container, which was then sealed with parafilm and exposed to a γ-radiation (Co^{60}) source at the Institute for Agrotechnological Research in Wageningen, with a dose rate of 14,8 Gy /s. The duration of the exposure and the distance from the radiation source were varied in different experiments to provide four different total doses of radiation. The pollen was applied immediately after irradiation to stigmata of flowers emasculated 0-2 days before.

Phenotypic selection for mutants in M_1

M_1 seeds from each fruit were sown separately in trays and three to four weeks later the seedlings were scored for the *tl, yv* and *c* phenotypes. At this stage, the M_1 plants were transferred to pots with sandy soil and tested for resistance/susceptibility to root-knot nematodes.

Nematode test

Second stage juveniles (J2), from a *Meloidogyne incognita* population grown on susceptible tomato plants, were kindly provided by Mr. Jan van Bezooyen (Department of Nematology, WAU). Between 1500 and 2000 J2s were applied per pot in holes made around the roots of each M1 plant and several parental line plants (VFNT Cherry and the W607 x Moneymaker hybrid) that served as controls. Each time the nematode test was performed care was taken, at least during the first three days after inoculation, to keep the soil temperature below 28°C and the moisture content optimal.

Six weeks after inoculation, the roots of the plants were inspected for the presence of galls. In our hands the root-knot nematode resistant test always gave clear plus/minus results in that the roots of the susceptible plants were studded with galls and the roots of resistant plants were clean.

DNA isolation and molecular markers

For Southern analysis, plant DNA was isolated from leaves as previously described (van der Beek *et al.* 1992). PCR analysis was carried out on DNA samples prepared as described by Wang and Cutler (1993). The SCAR marker REX-1 was developed by Williamson *et al.* (1994); SCAH13 is a SCAR marker developed by R. Weide (personal communication). For the *Aps-1* marker primers were designed using the sequence information from a genomic *Aps-1* clone (Aarts *et al.* 1991). Forward primer: 5'-ATGGTGGGTCCAGGTTATAAG-3'

Reverse primer: 5'-GATTGGCACAAGCTCATTCTG-3'. The amplified fragments were restricted with TaqI to reveal a *L. esculentum/L. peruvianum* polymorphism. The sources of the RFLP markers CD14, GP79, GP164, H4H10, H5G4, H6A2c2, LC216, RC8 and TG231, used in this work, were described by Weide *et al.* (1993).

Results and discussion

The root-knot nematode susceptible (*mi/mi*) hybrid (W607 x Moneymaker) which is heterozygous for the morphological markers *tl*, *yv* and *c* on chromosome 6 was pollinated with γ-irradiated pollen from the nematode resistant (*Mi/Mi*) line VFNT Cherry carrying the wild type alleles of the marker genes. Gamma irradiation-induced mutations affecting the wild type alleles were identified on the basis of pseudodominance of one or more of the four phenotypic markers *tl*, *yv*, *c*, and *mi*.

Effect of the dose of irradiation on the yield of mutants

To establish an irradiation dose with a high mutagenic effect and yet without deleterious effects on the M_1 seed set and seed germination, we varied the irradiation dose around the effective dose of 50 Gy reported in the literature (Khush et al. 1964; Van Wordragen et al. 1994). Furthermore, different doses of radiation can be expected to induce different spectra of chromosomal mutations. For example, at higher doses the probability of inducing double breakpoints by two hits within a small distance on the chromosome will be higher. As a result, at high doses a higher number of interstitial deletions is expected compared to terminal deletions. Four different doses of γ-irradiation varying from 50 Gy to 125 Gy were applied (Table 1, Fig. 1). An increase of the dose was associated with a consequent drop in the number of M1 seeds per fruit and the germination percentage of the seeds (Fig. 1). Pollination with VFNT Cherry pollen that had been treated with 125 Gy resulted in such a low seed setting and germination that only 12 M_1 plants were obtained (Table 1).

A comparison between the effects of radiation doses of 50 Gy and 100 Gy revealed that, although the same number of flowers were pollinated and a similar number of mutants was obtained, the total number of M1 plants to be screened differed significantly (Table 1). As a result, the calculated mutation frequency was six times higher at 100 Gy than at 50 Gy. With the highest mutation frequency at the investigated loci and relatively good seed set and germination rate (Fig. 1), a dose of 100 Gy appeared optimal for producing pseudodominant mutants.

Table 1. Number of pseudodominant, γ irradiation induced mutants at the chromosome 6 loci *Tl, Yv, C* and *Mi* in two M1 populations.

Dose	Experiment I					Experiment II						Total number
(Gy)	Number of M1 plants	mutants *tl*	*yv*	*c*	*tl,mi*	Number of M1 plants	mutants *tl*	*yv*	*c*	*mi*	*tl,mi*	of mutants
50	2277	0	0	0	5	1359	1	1	0	5	2	14
75	nd	-	-	-	-	515	0	0	0	1	2	3
100	330	0	0	0	4	559	1	0	0	3	5	13
125	nd	-	-	-	-	12	0	0	0	0	0	0
total	2607	0	0	0	9	2445	2	1	0	9	9	30

In experiment I only the *tl* plants were tested for nematode resistance, as were all plants in experiment II. nd = not done

Isolation of the mutants

A total of 5052 M1 plants from the cross of F_1 (W607 x Moneymaker) x VFNT Cherry (pollen-irradiated) were examined for pseudo-dominance of the morphological markers *tl, yv* and *c*, amongst which 20 phenotypically *tl* individuals (subsequently referred to as "mutants") and one *yv* mutant were recovered (Table 1). No plants showing the *c* phenotype, nor *tl, yv* double mutants, were found. Of the same M_1 plants, 2445 individuals were subjected to a nematode resistance test yielding nine nematode susceptible mutants. No phenotypes with intermediate nematode susceptibility were observed in this M_1 generation.

The closely linked loci *tl* and *Mi* on the short arm of chromosome 6 (Van Wordragen *et al.* 1994, 1996) showed similar mutant frequencies per chromosome, $7.9x10^{-3}$ and $7.4x10^{-3}$ respectively. When the *tl* mutants were subjected to a nematode resistance test, all but two turned out to be nematode susceptible indicating that the mutation included both the *tl* and *Mi* loci. This was later confirmed by molecular marker analysis.

For the *yv* locus, a relatively low mutation frequency of $0.4\ x10^{-3}$ was found, suggesting that only rather small interstitial deletions of the long arm of chromosome 6 are viable or had been transmitted through the pollen (Khush and Rick 1968). The total absence of *c* mutants in our M_1 population is difficult to explain. The *c* gene is located on the distal end of the long arm of the chromosome (see Fig. 2 A) and has been shown to be encompassed by terminal deletions (Khush and Rick 1968) though at a low frequency (Van Wordragen *et al.* 1996).

As not a single pseudodominant *tl, yv* double mutant was found among the 5052 M_1 individuals, deletions including *CEN 6* are clearly not viable (Khush and Rick 1968).

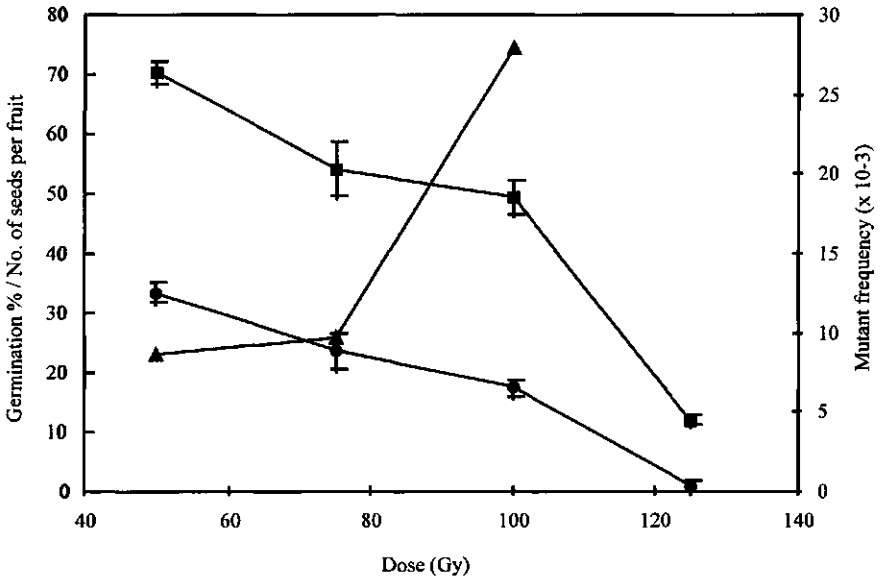

Fig. 1. Effect of the dose of γ-irradiation (Gy) on the seed setting (number of seeds per fruit, circles), germination rate (%, squares) and on the frequency of mutation for the *Tl* locus (per total number of scored M_1 plants, triangles). Vertical bars represent standard errors of the means.

Molecular-marker analysis of the mutants

Molecular markers polymorphic for *L. esculentum / L. peruvianum* were used to verify the genetic background of each of the mutant plants and to define the extent of the deletions.

All 29 *tl* and *mi* pseudo-dominants were found by PCR analysis to contain the *L. peruvianum Aps-1*^{l} allele, which resides on the long arm of chromosome 6 in tight linkage to the *yv* locus (Fig. 2 A) (Khush and Rick 1968; Van Wordragen *et al.* 1996). The selected *yv* mutant was tested with markers REX-1, LC216 and H5G4 that are located on the short arm of chromosome 6. Indeed, the *yv*-deletion plant showed the presence of *L. peruvianum* alleles of these loci (Fig. 2 B), indicating that this plant carries an interstitial deletion.

The nature and the extent of the mutations was then determined by analyzing the 29 *tl* and/or *mi* plants with appropriate RFLPs and SCARs from the short arm of chromosome 6 (Fig. 2 B). None of the mutant plants carried the *L. peruvianum* allele of the most distal marker LC216b and of the *tl*-linked marker H5G4, suggesting that they all contain terminal deletions of the short arm of chromosome 6. The two *tl* plants which were nematode resistant displayed the *L. peruvianum* alleles for the other tested molecular markers on the short arm.

Analysis of the *tl* and *mi* plants with the markers located proximal to the *CEN 6*, revealed deletions of various sizes, with most of the breakpoints (ten) near *CEN 6*. The markers LC216a and H4H10 have hitherto been found linked to the *yv* cluster (Ho *et al.* 1992; Van Wordragen *et al.* 1996) suggesting a location below *CEN 6* on the long arm (Van Wordragen *et al.* 1994). Since a number of *tl* and *mi* deletion plants from our study were lacking the *L. peruvianum* allele of LC216a and H4H10 (Fig. 2B), these markers are actually located on the short arm and not on the long arm as suggested by linkage analysis.

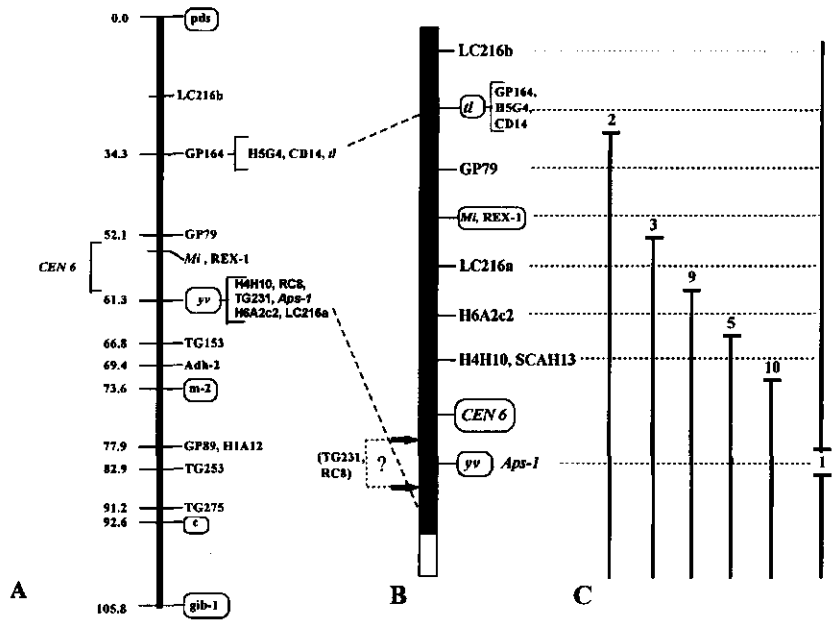

Fig.2. A. Integrated classical and molecular map of chromosome 6 after Weide et al. (1993). B. Molecular marker order as established by mapping of deletions induced on VFNT Cherry pollen. The black bar indicates the extent of *L. peruvianum* introgression in VFNT Cherry. C. The ciphers indicate the number of individual mutants of this kind.

Clearly, the deletion mapping approach was successful in resolving the order of markers that were refractory to mapping by genetic linkage analysis. The RFLP marker H6A2c2 was also resolved from the *yv* cluster (Weide *et al.* 1993) and found to be located on the short arm of chromosome 6, in agreement with recent data from Van Wordragen *et al.* (1996). The marker SCAH13b, that was originally identified as centromere-linked. (Rob Weide, in preparation), was deleted in the same plants in which H4H10 was deleted.

In the *yv*-deletion mutant, only one marker (*Aps-1*) from the *yv* cluster did not show the *L. peruvianum* allele. Apparently, this plant carries a small, interstitial deletion on the long arm of chromosome 6. The other markers from the cluster that were tested, RC8 and TG231, can be located on either side of the *yv-Aps-1* loci.

In summary, most of the mutants obtained by pollen irradiation have originated from terminal deletions of various size with breakpoints not being randomly distributed (Fig. 2 C). No correlation was found between the applied dose of radiation and the type of mutation and/or the location of the deletion breakpoints. The majority of deletions included both loci, *tl* and *Mi* and the *Mi*-linked marker REX-1. Recent cytogenetic studies in our laboratory indicate that REX-1 is located on the short arm of chromosome 6 within the euchromatin, but very close to the border of the centromeric heterochromatin (Zhong *et al.*, in preparation). This finding, in combination with the low recombination frequency encountered between REX-1 and *CEN 6* (Liharska *et al.* 1996), suggests that the markers LC216a, H6A2c2, SCAH13b and H4H10 must be located in the heterochromatin. Thus, most of the induced deletions in chromosome 6 are the result of breakpoints within the heterochromatin on the short arm. This non-random distribution of viable breaks has been earlier described for X-ray mutagenesis (Khush and Rick 1968). The apparent preference for breaks within the centromere proximal heterochromatin should be viewed in context with the question as to how chromosomes with terminal deletions can survive without a proper end, the telomere (Blackburn 1991). Possibly, the terminal deletions originate from two breaks, one near but not including the telomere. Alternatively, a mechanism may exist to heal the broken ends of the chromosome by the *de novo* addition of telomeric sequences, as shown for wheat (Werner *et al.* 1992). Heterochromatin might be the place where such a mechanism operates most efficiently.

Transmission of mutations

All 30 deletion-containing plants from this study produced flowers, with the single *yv* mutant as well as 18 of the 29 *tl* and *mi* plants providing viable seeds. M_2 generations of these mutants were raised, consisting of 40-100 individuals. In the progenies of the *tl* mutants only plants with a *tl/yv* phenotype were found, indicating that none of the deletions had been transmitted. In the M_2 generations of four *mi* mutants, 6-10 plants were selected for molecular-marker analysis. Tests with the PCR markers REX-1, SCAH13b and *Aps-1* revealed that in all plants only the *L. esculentum* alleles of these markers were present, indicating the absence of the mutated chromosome 6. These results support previous findings in maize, tomato, *Petunia* and *Arabidopsis* showing that the transmission of chromosomes

with large terminal deletions is very low (Stadler and Roman 1948; Khush and Rick 1968; Gerats *et al.* 1984; Timpte *et al.* 1994; Vizir *et al.* 1994).

The M_2 progeny (146 individuals) of the *yv* mutant consisted of 109 *yv* and 37 *yv*, *tl* plants indicating a normal 3:1 segregation ratio ($\chi^2_{3:1}$ = 0.01, P> 0.05) and thus a normal transmission of the chromosome carrying the deletion. Ten randomly selected *yv*-progeny plants tested with the *Aps-1* marker appeared to contain the *L. esculentum* allele of *Aps-1*, showing they were heterozygous for the *Yv* deletion. However, due to the relatively small sample tested, we can not exclude the possibility that genotypes homozygous for the *yv-Aps-1*^{1} deletion are viable.

Conclusions

This report describes the induction of deletions in a specific region of the tomato genome and their use for fine genetic mapping with molecular markers. The application of different doses of γ-radiation resulted in the same type of mutations, that is mainly large terminal deletions with breakpoints predominantly located in the pericentromeric heterochromatic region. The number of deletion mutants isolated in this study was helpful in resolving a cluster of molecular markers that may serve in the molecular cloning of *CEN 6*. On the other hand, the highly non-random distribution of the deletion's breakpoints will be a serious limitation in constructing a detailed locus order map in other regions of the plant genome.

Acknowledgments

We thank Jan van Bezooyen for supplying the nematodes and Henk Kuiper and his greenhouse staff for their concern and help which was instrumental to these experiments.

Chapter 4

GENETIC AND PHYSICAL LOCALIZATION OF THE ROOT-KNOT NEMATODE RESISTANCE LOCUS *Mi* IN TOMATO

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As part of a map-based cloning strategy to obtain the root-knot nematode resistance gene Mi, *tomato F_2 populations were analyzed to identify recombination points close to this economically important gene. A total of 21,089 F_2 progeny plants were screened using morphological markers. PCR-based flanking markers were developed and used to screen an additional 1887 F_2. Fine mapping of recombinants using newly developed AFLP markers and RFLP markers derived from physically mapped cosmid subclones allowed localization of* Mi *to a genomic region of about 550 kb. The low frequency of recombinants indicated that recombination was suppressed in these crosses. To circumvent the repressed recombination, a population of* L. peruvianum, *the species from which* Mi *was originally introgressed, that was segregating for resistance was developed. Screening of this population with PCR, RFLP and AFLP markers identified several plants with recombination points near* Mi. *Recombination frequency was approximately 20-fold higher in the* Mi *region of the* L. peruvianum *cross. Fine mapping of recombination points allowed further localization of* Mi. *By combining data from the* L. esculentum *and* L. peruvianum *recombinant analyses, it was possible to localize* Mi *to a region of the genome of less than 65 kb.*

This chapter is in press in *Molecular and General Genetics*

Introduction

Root-knot nematodes (*Meloidogyne* spp.) are endoparasites of thousands of crop species and are important pests of tomato worldwide (Williamson and Hussey 1996). The disease is characterized by the presence of galls or root-knots on infested plants. Symptoms include poor fruit yield, stunted growth, wilting, and susceptibility to other pathogens. Resistance to root-knot nematodes was introduced into the cultivated tomato, *Lycopersicon esculentum*, from the wild tomato species *L. peruvianum* by hybridization and embryo rescue (Smith 1944). Resistance is mediated by a single, dominant gene called *Mi*. *Mi* confers resistance against three major *Meloidogyne* species that infect tomato. *Mi*-mediated resistance is characterized by a rapid, localized necrosis or hypersensitive response near the anterior of the invading nematode (Dropkin 1969a; Ho *et al.* 1992). This response is triggered within 12 hr of infection.

Mi has been localized to the short arm of chromosome 6 (Fig. 1A). This chromosome has been mapped in considerable detail, and multiple markers linked to *Mi* have been identified (Klein-Lankhorst *et al.* 1991; Messeguer *et al.* 1991; Ho *et al.* 1992; Williamson *et al.* 1994). The abundance of polymorphisms between *L. esculentum* and *L. peruvianum* was helpful in developing a detailed map of the *Mi* region. In these studies, it was found that the nematode resistant tomato line Motelle and related lines retained only a small introgressed region from *L. peruvianum*. The physical size of the introgressed region has been estimated and delimits *Mi* to a 650 kb region of the genome (Vos *et al.*, in preparation). An RFLP marker, LC379, and a PCR marker, REX-1, have been identified within the 650 kb region (Ho *et al.* 1992; Williamson *et al.* 1994). Because of its tight linkage to *Mi*, REX-1 has proved to be a useful tool for monitoring the incorporation of *Mi* in breeding programs.

Recently, several plant pathogen resistance genes have been cloned using map-based cloning strategies (Martin *et al.* 1993; Bent *et al.* 1994; Mindrinos *et al.* 1994; Song *et al.* 1995; Dixon *et al.* 1996; Cai *et al.* 1997). Because of the importance of root-knot nematodes as pathogens and the broad host range of this pathogen, *Mi* is a particularly attractive cloning target. Yeast artificial chromosomes (YACs) spanning the 650 kb region to which *Mi* is localized have been identified (Vos *et al.*, in preparation). However, despite the availability of a physical map and markers spanning *Mi*, further localization of the gene has not been feasible due to lack of recombinants. Severe recombination suppression occurs in the *Mi* region of *L. esculentum* lines carrying the introgressed DNA (Messeguer *et al.* 1991; Ho *et al.* 1992; Ganal and Tanksley 1996; Liharska *et al.* 1996). This repression is thought to be a consequence of

the alien origin of the DNA segment carrying *Mi*. Proximity to the centromere may additionally contribute to the repressed recombination.

In this paper we present results of two strategies to identify and map additional recombination points near *Mi*. One strategy was based on large scale screening for recombinants between visible or PCR-based markers flanking *Mi* in *L. esculentum*. The second approach was to generate and analyze a segregating population of the wild tomato species *L. peruvianum* which was the origin of *Mi*. *L. peruvianum* is a heterogeneous species with accessions and individuals within accessions differing in marker alleles as well as in nematode resistance (Miller and Tanksley 1990; Yaghoobi *et al.* 1995). Several different root-knot nematode resistance genes have recently been identified within accessions of this species (Cap *et al.* 1993; Yaghoobi *et al.* 1995; Veremis and Roberts 1996b; Veremis and Roberts 1996c). We developed a population of plants segregating for resistance due to *Mi* and identified recombinants with cross-overs near *Mi* by using PCR-based flanking markers. Both approaches contributed toward localizing *Mi* and, by combining recombination analyses from all crosses, we have been able to localize *Mi* with newly developed molecular markers to a region of the genome corresponding to a physical distance of about 65 kb. Data indicating that the position of crossovers are unevenly distributed within this region are presented.

Materials & methods

Plant material

Development of populations for morphological screens from crosses between nematode resistant lines (83M/R, Motelle, Ontario-7620 and VFNT Cherry) and nematode susceptible line W607 (*tl yv/tl yv*) have been described previously (Liharska *et al.* 1996). Generation of the F2 population from the cross between VFNT cherry (*Mi*, *Aps-1*^{1}) and LA55 (*mi*, *Aps-1*$^{+}$) has also been described previously (Ho *et al.* 1992). *L. peruvianum* accession PI128657, the original source of *Mi* was obtained from USDA-ARS Germplasm Resources Unit, Geneva, N.Y. The susceptible *L. peruvianum* accession LA2964 was obtained from C. Rick, UC Davis.

Markers

GP79L: A 200 kb YAC hybridizing to potato marker GP79 (Gebhardt *et al.* 1991; Klein Lankhorst *et al.* 1991; Ho *et al.* 1992) was obtained from a VFNT cherry tomato library (Van Daelen 1995; Bonnema *et al.* 1996). Yeast miniprep DNA was extracted according to Hoffman and Winston (1987). A fragment of the YAC insert adjacent to the left arm of the vector was isolated by plasmid rescue (Schmidt *et al.* 1996). A 2.3 kb *Eco*RI insert was subcloned into pBluescript II SK+ (Stratagene, La Jolla, CA, USA) and DNA sequences were obtained for both ends. Sequences near the ends were selected to design the synthetic oligonucleotides used as STS (Sequence Tagged Site) primers: GP79L R1, 5' CACTCAATGGGGGAAGCAAC 3'; GP79L U2, 5' AATGGTAAACGAGCGGGACT 3'.

Aps-1: Primers were designed using sequence information from a genomic clone of *Aps-1* (Williamson and Colwell 1991). They were Aps-1 F2, 5' GGAACGTGGGTAGCATATGA 3'; Aps-1 R2, 5'

GCCAATGCTCATCAATGTGA 3'. *Taq*I restriction enzyme polymorphisms were revealed by digesting 10 µl aliquots of the amplified reaction with 5 units *Taq*I (New England Biolabs, Inc.) at 65°C. Digestion products were separated on 1.5% agarose gel and visualized by ethidium bromide staining.

C8B: C8B was originally identified as a RAPD marker (Weide *et al.* 1993). For this work it was converted to an STS marker with the following primers: F, 5'TACCCACGCCCCATCAATG 3'; R, 5'TGCAAGAGGGTGAATATTGAGTGC 3'. These primers amplified a single band that showed size polymorphism between LA55 and VFNT and could be used directly in screening *L. esculentum* crosses.

IK379: LC379 is a cDNA clone corresponding to a member of a large gene family. Among the bands detected by this probe is a 1.1 kb *Eco*RI fragment linked to *Mi* (Ho *et al.* 1992). To clone the 1.1 kb *Eco*RI fragment, DNA from a nematode resistant tomato line was digested with *Eco*RI and size-fractionated on an 0.8% agarose gel. The region of the gel with 0.8-1.4 kb fragments was cut out and the DNA eluted using Geneclean (Bio 101, Inc., La Jolla, CA, USA). The DNA was ligated into the *Eco*RI site of phage lambda gt 11 (Stratagene, La Jolla, CA, USA) following manufacturer's recommendation . Following three rounds of screening a single phage was isolated that carried a 1.1 kb *Eco*RI fragment hybridizing to LC379. The 1.1 kb fragment was subcloned into pBluescript II SK+ and was referred to as IK379. This was used as a probe for Southern blots.

To generate AFLP markers, DNA was isolated as described by Stewart and Via (1993). Genomic DNA was digested with restriction enzymes *Pst*I and *Mse*I, and AFLP markers were identified (Vos *et al.* 1995); Vos *et al.*, in preparation). Markers were designated "PM+numerical code."

PCR analysis

PCR was performed on tomato DNA extracted following methods described by Wang *et al.* (1993). The reaction was carried out in a volume of 25-50 µl in the presence of 0.5 mM of each of the primers, 0.2 mM dNTPs (Pharmacia) in a buffer containing 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl2, 1.0% Triton X-100, and 1.25-2.5 units of *Taq* polymerase (Promega). Reactions with *L. peruvianum* DNA template contained 0.01% gelatin in the buffer. Cycling conditions were 94°C for 30 s, 58-60°C for 1 min, and 72°C 2 min for 30 cycles, followed by a 10 min extension at 72°C. For *Aps-1* primers with *L. peruvianum* DNA template, 55°C annealing temperature was used.

Screening for recombinants

For *L. esculentum* populations to be screened with PCR markers, seeds were germinated in vermiculite in styrofoam flats with 2.5 cm x 2.5 cm wells and maintained at 22-26°C and 16 h light period. A small leaflet from two-week-old seedlings was extracted for PCR analysis. Recombinants were transplanted into 1L pots and maintained in a greenhouse. Cuttings were made for nematode screens. Seeds were collected from the heterozygous recombinants when necessary to produce homozygous plants for screens.

For screening *L. peruvianum* populations with PCR markers, seeds were germinated in wells (15 mm diameter) of microtiter plates (24-well), 3/4 filled with solidified 1% agarose in 1X MS medium. Plates were capped, marked and put in clear plastic boxes with covers. Boxes were put in a growth chamber at 24°C with a 16 h light period. After about 3 weeks, a small leaflet was extracted for PCR analysis. Recombinants were transferred into soil in magenta boxes, and maintained in a growth chamber for 3-4 weeks. Later, plants were transplanted into 1l pots and maintained in a greenhouse. Cuttings were made for nematode assays. Greenhouse grown plants were used for screening with AFLP markers.

Nematode screen

Second-stage juveniles (J2) of *Meloidogyne javanica* VW4 collected from an hydroponic culture (Lambert *et al.* 1992) or J2 collected from *M. incognita* infected tomato roots (kindly provided by J. van Bezooyen, Dept. of Nematology, WAU) were used as inoculum. Six-to-eight-weeks-old plants or well rooted cuttings grown in river sand were infected with 2000-3000 J2 and maintained in a greenhouse at 22-26°C. Nematode resistance assays were performed as previously described (Yaghoobi *et al.* 1995; Liharska *et al.* 1997).

RFLP analysis

DNA extractions were performed as described (Williamson and Colwell 1991; Van der Beek *et al.* 1992). Genomic DNA (5-10 µg) was digested with appropriate restriction enzyme using manufacturer's buffers in 50 µl at the recommended temperature overnight. Digested DNA fragments were separated on 0.8% agarose gels and Southern blotted onto Nytran plus membrane (Schleicher & Schuell) (Sambrook *et al.* 1989) or Hybond N+ (Amersham Co., Arlington Heights, Ill. USA). DNA was linked to the membrane by exposure to 120mJ of UV using a Spectrolinker XL-1500 (Spectronics) or by heating the membranes at 80°C for an hour. Probes were prepared from inserts isolated from agarose using Geneclean or amplified from a single bacterial colony using PCR (Ho *et al.*, 1992). Probes were labeled with α-^{32}P-dCTP by random priming method (Feinberg and Vogelstein 1983) using a multipriming labeling kit (Amersham Co.). Hybridization were carried out overnight either in 50% formamide at 42°C or in aqueous solution at 65°C. Final washes were done in 0.2-0.5 x SSC, 0.1% SDS at 65°C.

Results

Molecular markers in the Mi region

To expedite screening for recombinants, key markers flanking *Mi* were converted into PCR-based forms which could be used as STS or CAPS (Cleaved Amplified Polymorphic Sequence) markers. The gene *Aps-1*, which maps below *Mi* (Fig. 1) had previously been cloned and sequence information was available (Aarts *et al.* 1991; Williamson and Colwell 1991). Selected primers amplified a 1.9 kb fragment from both resistant and susceptible individuals. The enzyme *Taq*I cleaved the band from the resistant plant into two fragments and that from the susceptible plant into three fragments.

GP79 is a potato DNA probe that had previously been mapped as an RFLP marker above *Mi* on chromosome 6 (Klein-Lankhorst *et al.* 1991) (Fig. 1A). A subclone obtained from the left end of a YAC clone from a VFNT cherry tomato library that carried the tomato homolog of GP79 was used to develop PCR marker GP79L. GP79L primers amplified a single band in LA55 and 2 bands in VFNT, one the same size as in LA55 and the other larger. The scorable band behaved as dominant marker, but genotypes homozygous and heterozygous for this band in VFNT could usually be distinguished by relative intensity of the two bands.

Physical mapping using YACs that span the *Mi* region showed that this marker mapped between markers IK379 and REX-1. This marker is designated GP79L-I (Fig. 1B).

AFLP markers (Vos *et al.* 1995) near and within the 650 kb region were generated by screening DNA from tomato lines nearly isogenic for *Mi*. These markers, designated by PM and numerical identifier (PM03, PM19, etc.) were positioned in the region by analysis of YAC clones and cosmid subclones spanning the *Mi* locus (Vos *et al.*, in preparation). Positions of key AFLP markers used in this analysis are indicated in Fig. 1.

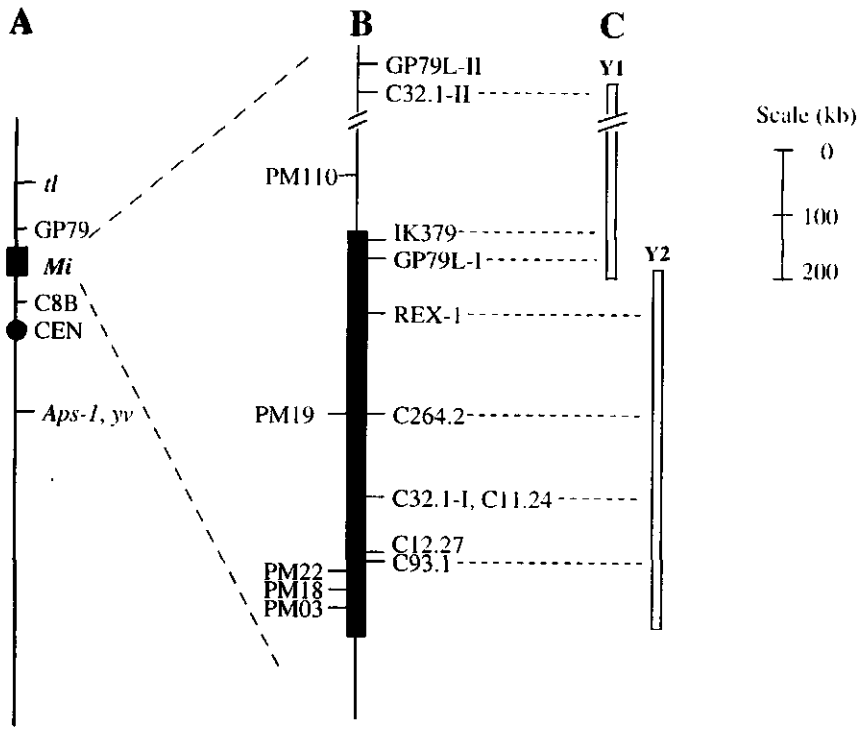

Fig. 1. Map of the *Mi* region of Chromosome 6. A. Chromosome 6 with flanking markers (Ho *et al.* 1992; van Wordragen *et al.* 1994). B. Expanded view of the *Mi* region. The thick bar represents the approximately 650 kb introgressed region present in the line Motelle. The positions of key markers are indicated. The 'PM' markers to the left of the bar are AFLPs (Vos *et al.*, in preparation). The markers on the right are RFLP markers that have been physically positioned in the *Mi*-region (this work; Vos *et al.*, in preparation).

Additional RFLP markers more precisely positioned within the *Mi* region were obtained as follows: YAC #1256, which spanned most of the 650 kb *Mi* region (Vos *et al.*, in preparation), was subcloned into cosmids. The relative position of these cosmids was determined using AFLP markers and pulsed field electrophoresis of digested YACs. *Eco*RI-*Hind*III fragments were subcloned from selected cosmid clones. These subclones were tested for their ability to identify low copy number sequences by screening Southern blots with genomic DNA digests from tomato lines VFNT cherry (*Mi/Mi*) and Moneymaker (*mi/mi*). Polymorphisms between the two lines were obtained with many of the restriction enzymes tested and those which gave the clearest polymorphisms are listed in Table 1.

Table 1. RFLP probes used for mapping *Mi*.

	Restriction Enzyme for Polymorphism	
Probes	*L. esculentum* crosses	*L. peruvianum* crosses
C264.2	*Dra*I, *Eco*RI	*Hae*III
C32.1	All	*Hae*III
C11.24	-	*Hae*III
C12.27	*Eco*RI, *Hind*III, *Taq*I	none
C93.1	*Eco*RI, *Eco*RV, *Xba*I	*HindIII*

Probes, derived from cosmid subclones, were tested on *L. esculentum* DNA of parental types digested with the restriction enzymes *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, *Nsi*I, *Taq*I and *Xba*I. *L. peruvianum* parental DNA was digested with *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hind*III, *Hinf*I, *Mbo*I. Those enzymes which gave the clearest polymorphisms are listed. "All" indicates that all enzymes tested gave clear polymorphisms. None indicates that no useful polymorphism was identified.

Identification of recombination events near Mi in L. esculentum using flanking markers

Recombination events near *Mi* were identified in *L. esculentum* F_2 populations by screening with either morphological or PCR-based flanking markers. The first class of recombinants was identified from crosses involving the morphologial markers *yv* (yellow virescence) and *tl* (thiamineless) which flank *Mi* (Fig. 1A). In F_2 populations derived from crosses between nematode resistant tomato lines (tl^+ yv^+) and the nematode susceptible line W607 (*tl yv*), the recombinant classes *tl* yv^+/*tl yv* and tl^+ *yv*/*tl yv* were identified phenotypically at the seedling stage (Liharska *et al.* 1996). A total of 21,089 F_2 progeny plants were scored, and 489 recombinants between *tl* and *yv* were identified. Of these, 318 were tested for nematode resistance and analyzed with the PCR marker REX-1 (Williamson *et*

plants were scored, and 489 recombinants between *tl* and *yv* were identified. Of these, 318 were tested for nematode resistance and analyzed with the PCR marker REX-1 (Williamson *et al.* 1994) and the RFLP marker IK379, which is located at the centromere-distal border of the Motelle *Mi* region. Most of the recombinants (228 plants) were found above marker IK379 and only 88 were below this marker. One plant, MT107, had a recombination between REX-1 and IK379. However, this plant died before it could be tested for nematode resistance. We chose for additional marker analysis only the *tl* or *yv* plants with recombination below IK379, which is very close to the border of the introgressed region, and for which a nematode resistance phenotype could be scored without progeny testing. In total, 64 recombinants of this kind were screened with AFLP markers, PM110 and PM03 which flank the *Mi 1* region (Fig. 1). From this analysis, two additional recombinants in the Motelle region, VT12 and VT14, were identified (Table 2).

The second class of recombinants was obtained by screening an F_2 population from a cross of VFNT cherry (*Mi/Mi*)and LA55 (*mi/mi*) (Ho *et al.* 1992) with PCR-based markers. GP79L-I, which maps near the upper end of the introgressed region was used as one marker. *Aps-1*, which maps below *Mi* was used as the second flanking marker to score a total of 1,887 F_2 seedlings. Nine individuals with recombination events between the GP79L-I and *Aps-1* loci were identified. It is possible that some recombinants were missed because of difficulty in some gels to distinguish heterozygotes and homozygotes for marker GP79L-I. These nine plants then were scored with PCR markers REX-1 and C8B to further localize the recombination points (Fig. 1). No recombinants were identified between GP79L-I and REX-1. Four plants had recombination points below C8B placing this marker above *Aps-1* on chromosome 6 (Fig. 1). One plant died, and the remaining 4 recombinants (I H7, VI G19, VIII G5, and X A8) were assayed for nematode resistance and analyzed in more detail (Table 2).

Fine mapping L. esculentum recombinants by RFLP markers

Cosmid subclones proved to be useful probes for fine mapping as they could be generated for specific regions of the physical map of *Mi*. For example, probe C93.1 is a single-copy, cosmid-derived probe that is positioned near the centromere-proximal end of the 650 kb introgressed region (Fig. 1). *L. esculentum* (e) and *L. peruvianum* (p) alleles can be distinguished on Southern blots restricted with either *Eco*RI or *Eco*RV (Fig. 2). With this marker, two recombinants, IH7and VIIIG5, were shown to have recombination points between C93.1 and C8B. Analysis of recombinant VIG19 indicated that the recombination point in this plant was above C93.1 and below *Mi*.

Table 2. Fine mapping of *L. esculentum* recombinants with markers flanking *Mi.*

Marker / Plant	PM110	IK379	GP79L-I	REX1	C264.2	C32.1-I	*Mi*	C12.27	C93.1 EcoRV	C93.1 EcoRI	PM18	PM03	C8B	*Aps-1*
LA 55	n	0	0	e/e	e/e	e/e	S	e/e	e/e	e/e	n	n	e/e	e/e
VFNT	n	p	p	p/p	p/p	p/p	R	p/p	p/p	p/p	n	n	p/p	p/p
Motelle	e/e	p	p	p/p	p/p	p/p	R	p/p	p/p	p/p	p/p	p	e/e	e/e
I H7	n	n	0	e/e	e/e	e/e	S	n	e/e	e/e	n	n	e/p	e/e
VI G19	e/e	n	0	e/e	n	e/e	S	e/p	e/p	e/p	n	n	e/p	e/p
VIII G5	e/p	n	p	e/p	e/p	e/p	R	n	e/p	e/p	n	n	e/e	e/e
X A8	p/p	n	p	p/p	p/p	p/p	R[a]	p/p	p/p	e/p	n	n	e/p	e/p
VT12	e/e	0	0	e/e	n	n	S	e/e	e/e	p/p	e/p	p	n	e/p
VT14	e/e	0	n	e/e	n	n	S	n	n	n	e/e	p	n	e/p
MT107	e/e	0	n	e/p	n	n	n[b]	n	n	n	e/p	p	n	e/p

e= allele found in nematode susceptible *L. esculentum*, p= *L. peruvianum*, 0= no specific fragment detected,

n= not determined, R= resistant, S= susceptible.

a Genotype is based on progeny testing.

b dead, therefore, no nematode assay was performed.

For recombinants VT12 and XA8, the allele of C93.1 that was observed depended on the restriction enzyme used for the analysis (Fig. 2). On a Southern blot, the recombinant VT12 produced the pattern expected for the *L. esculentum* allele (e/e) after digestion with *Eco*RV but the pattern expected for the *L. peruvianum* allele (p/p) after digestion with *Eco*RI. Conversely, recombinant XA8 displayed the *L. peruvianum* pattern after *Eco*RV digestion but was heterozygous (e/p) after digestion with *Eco*RI. Since C93.1 is a single copy sequence, the simplest explanation is that the recombination point in each case is close to C93.1 and lies between the *Eco*RI and *Eco*RV site on the centromere-distal side of the probe (Fig. 2). Double digest patterns supported this result and indicated that the recombination points in these two plants were within the same 5.3 kb region (Fig. 2C).

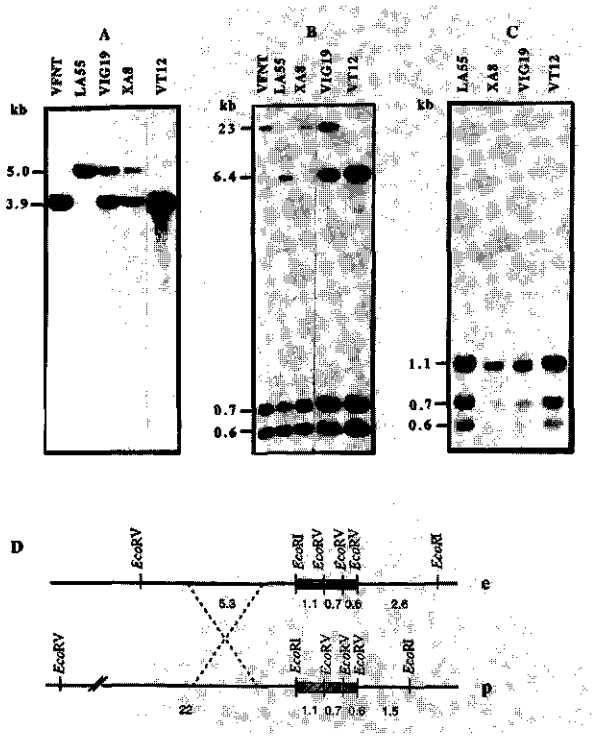

Fig. 2. Positioning of recombination points near marker C93.1. A. Southern blot of DNA from *L. esculentum* recombinants digested with *Eco*RI probed with c93.1. B. Southern blot digested with *Eco*RV. C. DNA digested with *Eco*RI and *Eco*RV. D. Diagram of the proposed *Eco*RI and *Eco*RV fragments hybridizing to the 2.4 kb probe C93.1 based on Southern blots. Numbers below the bars indicate fragment sizes in kb. Cross over points in recombinants XA8 and VT12 are localized to the 5.3 kb region marked by the 'X.' Position of the C93.1 sequence is indicated by the thick, cross-hatched bar. The letters 'e' and 'p' indicate *L. esculentum* and *L. peruvianum* chromatids, respectively.

Nematode resistance assays of VT12, XA8 and VIG19 indicated that *Mi* is located above C93.1 (Table 2). The recombination point of VIG19 was further localized above marker C12.27 which is positioned between 1kb and 20 kb above C93.1, indicating it is the closest to *Mi* on the centromere proximal side. This information excludes that *Mi* is located in the centromere-proximal 100 kb of the Motelle-introgressed region and localized *Mi* to a physical region of about 550 kb.

Identification of L. peruvianum recombinants in the Mi region

The data presented above support previous observations (Ganal and Tanksley 1996; Liharska *et al.* 1996) that recombination is severely repressed in the introgressed region carrying *Mi* in *L. esculentum* crosses. To circumvent the repressed recombination, we developed a cross between resistant and susceptible *L. peruvianum*. Plants from PI128657, the *L. peruvianum* accession that was the original source of *Mi*, were screened for nematode resistance, and a resistant individual, PI128657-G, was identified. Additional *L. peruvianum* accessions were screened to identify a susceptible source. From accession LA2964, a plant, LA2964-2, that was susceptible to nematodes and cross-compatible with PI128657-G, was identified and used to produce F_1 progeny. Because *L. peruvianum* is self-incompatible, it was necessary to mate sibs of the F_1 generation to generate a pseudo-F2 population segregating for nematode resistance.

For many RFLP and PCR markers, the alleles found in this *L. peruvianum* cross differed from those found in either *L. esculentum* or in the introgressed region from *L. peruvianum* present in nematode resistant *L. esculentum*. Thus, it was necessary to identify and characterize alleles of each marker to score progeny from this cross. We developed a PCR-based assay that distinguished *Mi*-flanking loci GP79L and *Aps-1*. With PCR primers for marker GP79L, DNA from PI128657-G and LA2964-2 each produced two different size bands. One of these bands was polymorphic in size between the parental lines and the other was constant. The F_1 progeny was heterozygous and each carried three bands; thus, all three genotypes could be scored in the pseudo-F_2 population for this marker. The polymorphic bands segregated as a single trait tightly linked to *Mi*. Segregation analysis (below and Table 3) indicated that this locus was positioned above GP79L-I; therefore we designated the locus GP79L-II.

With *Aps-1* primers, a band of the same size was amplified from both resistant and susceptible plants. Upon digestion with either *Sac*I or *Bam*HI, the band from the resistant parent was cleaved into two fragments while that of the susceptible was not cleaved. Because additional root-knot nematode resistance genes unlinked to *Mi* have been identified in *L.*

Table 3. Fine mapping of *L.peruvianum* recombinants with markers flanking *Mi.*

Markers / **Plant**	**GP79L-II**	**C32.1-II**	**PM110**	**PM19**	**C264.2**	**C32.1-I**	**C11.24**	***Mi***	**C93.1**	**PM22**	**Aps-1**
PI128657-G	1/1*	1/1	1/1	3/1	3/1	1/1	1/1	R	1/1	3/1	1/1
LA2964-2	2/2	0/0	2/2	2/2	2/2	2/2	0/0	S	2/2	0/0	2/2
JG0-6	1/2	1/0	1/2	3/2	3/2	1/2	1/0	S	2/2	0/0	2/2
KAS-201	1/2	1/0	1/2	3/2	3/2	1/2	1/0	S	2/2	0/0	2/2
KAS-1081	1/2	1/0	1/2	3/2	3/2	1/2	1/0	S	2/2	0/0	2/2
KAS-1284	1/2	1/0	1/2	3/2	3/2	1/2	1/0	R	2/2	0/0	2/2
KAS-241	2/2	0/0	1/2	1/2	1/2	1/2	1/0	R	2/2	0/0	2/2
KAS-84	2/2	0/0	2/2	1/2	1/2	1/2	1/0	R	1/2	1/0	1/2
KAS-1357	2/2	0/0	2/2	3/2	3/2	1/2	1/0	R	1/2	3/0	1/2
JG6-8	2/2	0/0	1/2	1/2	1/2	1/2	1/0	R	1/2	1/0	1/2

*Alleles associated with the resistant parent were designated '1' and '3' while those associated with the susceptible parent were designated '2' or, if no corresponding band was detected, '0'.

peruvianum (Cap *et al.* 1993; Yaghoobi *et al.* 1995; Veremis and Roberts 1996a), the F_2 progeny was assessed to confirm segregation of nematode resistance with *Mi*-linked markers from the resistant accession.

Individual seedlings from the pseudo-F_2 population were scored for *Aps-1* and GP79L-II. In the first round of screening 508 individuals were scored, and 50 plants with recombination events between *Aps-1* and GP79L-II were identified. Cuttings were prepared from 42 of these for further molecular analysis. An additional 390 F_2 plants from the pseudo-F_2 population (KAS001 to KAS390) as well as 390 progeny (KAS1001 to KAS1390) from a cross between F_2 plants that were heterozygous for resistance were screened using the AFLP markers PM110, PM19 and PM22. This analysis resulted in the identification of 23 recombinants between PM110 and PM22 which flank *Mi* (Fig. 1). Five of these recombinants could not be tested directly for nematode resistance as the recombinant chromosome was combined with a non-recombinant chromosome from a resistant parent; these were not studied further.

Fine mapping of L. peruvianum recombinants with RFLP's

To identify useful probes for fine mapping, Southern blots with DNA of resistant and susceptible parental and F_1 plants of the *L. peruvianum* mapping population digested with several different enzymes were probed with 8 subclones of cosmids from the *Mi* region. The most informative markers were those that distinguished both alleles in resistant lines from those originating from the susceptible parent, such as marker C264-2 (Fig. 3). Most cosmid subclones hybridized to multigene families with one or more family members unlinked to the *Mi* locus. Polymorphisms linked to *Mi* were identified by checking for co-segregation of the polymorphisms with the locus in subpopulations of F_2 plants (Fig. 3, for example).

Since cosmid subclone C93.1 produced a simple pattern and had been determined to map below *Mi* in the *L. esculentum* cross, this marker was used to screen the 42 recombinants between *Aps-1* and GP79L as well as the 18 recombinants between PM110 and PM22. Two recombinants, KAS 241 and KAS1284, put C93-1 below the *Mi* locus, supporting the gene order obtained from the *L. esculentum* studies. Analysis with RFLP probe C264-2, which has been physically positioned near the center of the 500 kb region to which *Mi* had been localized, was used next (Fig. 3). Restriction patterns from three recombinants, JG0-6, KAS201, and KAS1081, placed C264-2 above the *Mi* locus. As a result *Mi* is flanked by two RFLP markers, C93-1 and C264-2, and, thus, physically localized to a region of about 200 kb. The five recombinants with crossover between C264-2 and C93-1 and control plants were

screened with additional RFLP markers to produce a more detailed map of the *Mi* locus. Subclone C32-1 is physically positioned below C264-2 and about 65 kb above c93-1 in VFNT cherry tomato genome. This probe hybridized to a small gene family. One polymorphic member (C32-1-I) mapped below C264-2 based on recombination events in three plants, JG6-8, KAS84, and KAS1357. Another polymorphic member of this family, C32-1-II was genetically inseparable from marker GP79L-II and mapped above AFLP marker PM110, based on recombinant KAS-241 (Table 3).

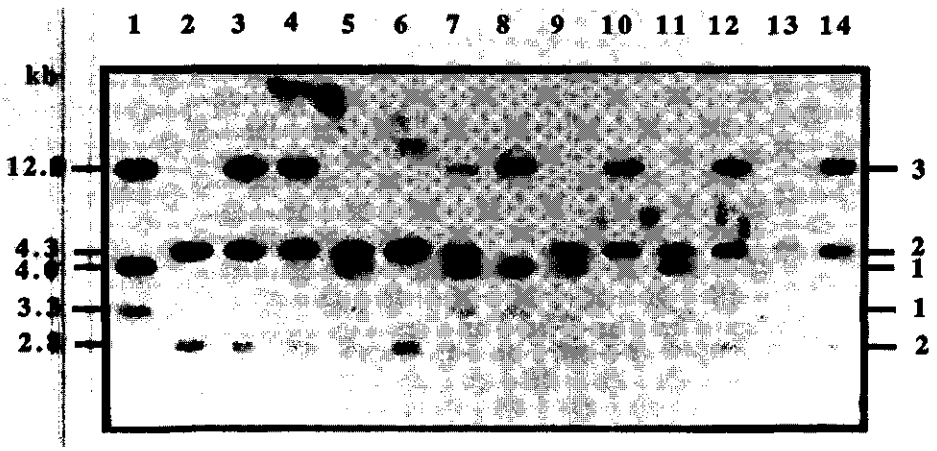

Fig. 3. Southern blot of *L. peruvianum* recombinants probed with C264. DNA was cleaved with HaeIII. Lane 1, contains DNA from the resistant parent PI128657-G; lane 2, the susceptible parent, LA2964-2; lanes 3-14 contain DNA from segregating F2 progeny. For each band, the fragment size is indicated to the left of the figure and the allele designation is indicated to the right. The two distinguishable alleles in the resistant parent are designated as '1' and '3'. The common allele in the susceptible parent is '2'. Linkage of segregating polymorphisms to *Mi* and other markers from the region was confirmed with this and other Southern blots.

The presence of two family members in the region and their positions were confirmed by hybridization to YACs spanning the *Mi* region (results not shown). Probe C11-24 which corresponds to a single copy fragment positioned 1 kb below C32-1-I (Bodeau *et al.* in preparation), produced a pattern corresponding to C32-1-I in the recombinant analysis, supporting the placement of *Mi* between this marker and the centromere. Out of the five recombinants between C11.24 and C93.1, three were susceptible and two were resistant to nematodes (Table 3), consistent with recombination points above and below *Mi*. We were not

able to obtain useful markers with any additional probes between these points and could not further localize *Mi*.

Taking the *L. esculentum* and *L. peruvianum* mapping data together, *Mi* was localized to the region between markers C11.24 and C12.27 which are separated by a physical distance of less than 65 kb in the nematode resistant line VFNT cherry (Vos *et al.*, in preparation; Bodeau *et al.*, in preparation).

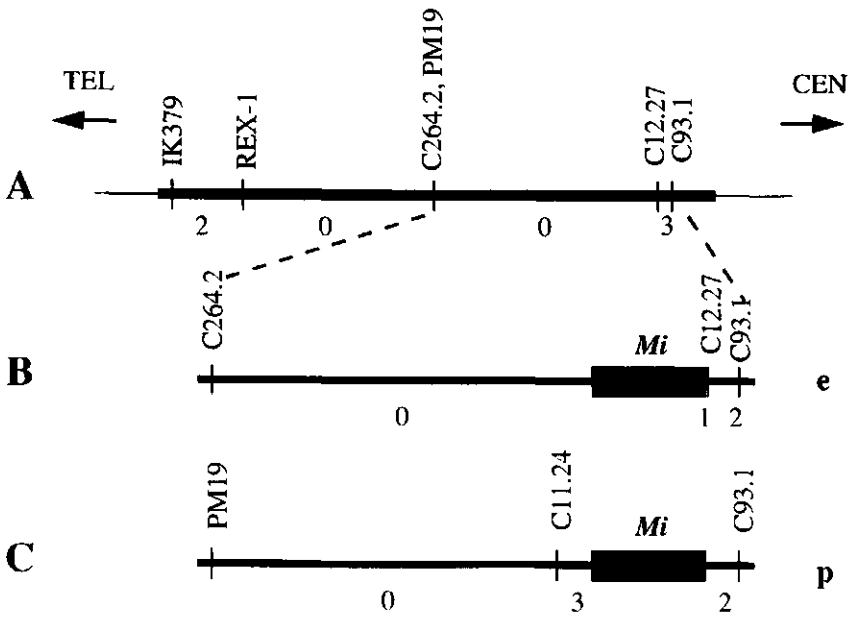

Fig. 4. Physical map and distribution of cross over positions. A. Represents the 650 kb introgressed region in the line Motelle. The number of *L. esculentum* crossovers identified in intervals between IK379 and C93 is indicated below the line labeled 'e'. B. An expansion of the C264 to C93 region and cross over points in *L. esculentum* cross. The region to which *Mi* has been localized is indicated as a black bar. C. The positions of the *L. peruvianum* crossovers in the same region (PM19 and C264.2 map to the same cosmid) is indicated below line 'p'.

Discussion

Fine mapping of the *Mi* region is a crucial step for obtaining a clone of this agronomically important gene. Suppression of recombination in the *Mi* region in *L.esculentum* crosses segregating for resistance has been a major limitation in obtaining a fine map of the region (Messeguer *et al.* 1991; Ho *et al.* 1992; Liharska *et al.* 1996). Messeguer *et al.* (1991) found a 5-fold lower recombination rate between *Mi*-flanking markers GP79 and *Aps-1* in *L.esculentum* crosses containing *Mi* compared with *L. esculentum/L. pennellii* crosses. Liharska *et al.* (1996) found a suppression of recombination of up to 6-fold between *tl* and *yv* in crosses segregating for *Mi*. This repressed recombination has been proposed to be due to lack of proper pairing between the introgressed *L. peruvianum* DNA and *L. esculentum*. In an attempt to circumvent this problem, we designed intraspecific crosses of *L. peruvianum* plants segregating for *Mi*. In these crosses we found that the recombination frequency in the *Mi* region is approximately 20-fold higher than in *L. esculentum* crosses containing *Mi*. This is in agreement with the findings of Ganal and Tanksley (1996) who found 15-fold more recombination in the *Mi* region for crosses within *L. peruvianum* than for crosses between resistant and susceptible *L. esculentum* lines. Analysis of the intraspecific cross therefore allowed a much more detailed map of *Mi* to be produced with a much smaller number of plants than would have been required for the *L. esculentum* crosses.

L. peruvianum is a heterogeneous species complex (Rick 1986; Miller and Tanksley 1990). Considerable polymorphism exists within, as well as among, accessions. For example, although the introgressed region from *L. peruvianum* in *Mi*-carrying *L. esculentum* lines was derived from accession PI128657, the same accession that was the source of our resistant *L. peruvianum* parent PI128657-G, we observed considerable polymorphisms in fragment sizes between these two lines in the *Mi* region. Nevertheless, when genetic maps of the region based on our recombination analysis were compared between *L. esculentum* crosses and *L. peruvianum* crosses, the marker order was consistent. We also found agreement between the genetically determined marker order and that in the physical map developed from the YAC contig of the *Mi* region. All recombination events that we investigated in the *L. peruvianum* intraspecific crosses could be explained by single crossovers, except for KAS241, which appears to be a double recombinant (Table 3). The ability to score for polymorphisms and for nematode resistance in the segregating progeny has allowed us to obtain a fine map and to localize the *Mi* gene. By combining data from *L. esculentum* and *L. peruvianum* crosses and utilizing different types of molecular markers, we have localized *Mi* to a region of less than 65 kb.

Our study provides the opportunity to compare recombination frequencies within a small region of the genome which has been physically, as well as genetically, characterized. A non-uniform distribution of the recombination points was found in the *Mi* region in the *L. esculentum* crosses (Fig. 4). All recombination events that have been detected in Motelle within the 650 kb region that carries *Mi* are localized to two regions. Two recombinants, VL628-19 (Ho *et al.* 1992) and MT107 (this work), have been identified between markers IK379 and REX-1. Three recombinants described in this work have crossover between markers C93 and *Mi*, a region of less than 80 kb. In fact, we present data that two independent recombination points are located within a 5.3 kb region. In contrast, no recombinants have been identified in the more than 300 kb region between these pairs of markers in the *L. esculentum* crosses. Thus, it appears that recombination in the region to the left of *Mi* is more severely repressed and that, even with larger number of recombinants, further localization of *Mi* would not have been possible in *L. esculentum* crosses segregating for resistance. The distribution of recombination points was also uneven, but less so, in the *L. peruvianum* cross (Fig. 4). We identified 5 recombination events between C93 and C11.24, a region of about 65 kb but none between markers C11.24 and PM19 which are approximately 150 kb apart. Unfortunately, we were not able to identify polymorphisms within the 65 kb region to refine the map position of *Mi*.

Other resistance genes, in addition to *Mi*, have been mapped in this region of the genome. An aphid resistance gene, *Meu1*, is present in lines that carry *Mi* and is located within 650 kb of *Mi* (Kaloshian *et al.* 1995). It is thought that *Meu1* was introgressed along with *Mi* from *L. peruvianum*. Two genes, *Cf2* and *Cf5* that confer resistance to specific races of the fungus *Cladosporium fulvum* have been mapped below GP79 and above the introgressed *L. peruvianum* region of Motelle (Dickinson *et al.* 1993; Dixon *et al.* 1995). The data in this paper indicate that the position of *Mi* is near the centromere-proximal end of the 650 kb introgressed region in Motelle. The positioning of *Mi* near the bottom of the introgressed region places it at least 400 kb from *Cf2* and *Cf5*. The *Cf2* locus has been isolated by map-based cloning and was shown to contain two almost identical genes, each conferring resistance to isolates of *C. fulvum* expressing the corresponding *Avr2* gene (Dixon *et al.* 1996). The two *Cf2* genes resemble the unlinked *Cf9* gene in that they carry a putative signal peptide, an extracellular leucine rich repeat (LRR) motif, and a C-terminal membrane anchor (Dixon *et al.* 1996). The localization of *Mi* to a 65 kb region of the genome now makes feasible positional cloning and complementation to obtain a clone of *Mi*, an approach similar to that used for cloning *Cf2*. Comparison of the structure and evolution of the resistance genes in this cluster should prove interesting.

Chapter 5

A SPONTANEOUS MUTATION IN A TOMATO GENOTYPE CAUSING LOSS OF RESISTANCE TO ROOT-KNOT NEMATODES AND POTATO APHIDS

Tsvetana Liharska, Jan Hontelez, Valerie M. Williamson, Ab van Kammen and Pim Zabel

A tomato genotype OT745 which has lost spontaneously resistance conferred by the tightly linked Mi-1 *(root-knot nematodes) and Meu1 (potato aphids) loci on chromosome 6 was studied. Test crosses of OT745 with* Mi-1*-resistant and* Mi*-susceptible lines revealed that the cause for the susceptible phenotype OT745 is a recessive mutation located within the* Mi-1 *introgression. Search for the mutation* lmm *(loss of* Mi-1 *and* Meu1*) was conducted using conventional and long range RFLP mapping. For this purpose cosmid clones distributed over the* Mi-1 *region were subcloned and employed as probes for Southern analysis with , in total, 23 restriction enzymes. For only one enzyme, Cla I, a polymorphism between OT745 and a* control Mi-1 *resistant line was found on PFGE blots. The Cla I polymorphic site was located and the genomic fragment containing the polymorphism was isolated from the resistant genotype. PCR combined with restriction analysis of the corresponding genomic fragments from OT745 and from a susceptible control revealed that in these genotypes the Cla I recognition site is hypermethylated. Our current interest is to localize the Cla I site relative to the two resistance genes and to further characterize the Cla I region.*

Introduction

Tomato (*Lycopersicon esculentum*) is a host for many phytopathogenic organisms, including viruses, fungi, bacteria, nematodes and parasites. The potential of plants to detect the presence of a particular pathogen and to rapidly activate a series of defense responses that eventually result in suppression of the pathogen development and reproduction, is defined as host resistance. This trait is under genetic control involving both plant encoded resistance gene(s) and their cognate pathogen avirulence gene(s). Sources of resistant germ plasm in tomato are often found in its wild relatives and resistance to some major pathogens has been introgressed in tomato over the years using interspecific crosses (Rick 1982). Besides of their economical importance, such breeding programs provide useful starting material for isolating resistance genes via positional cloning. Over the past few years efforts to isolate disease resistance genes have intensified and, to date, the structure and sequence of four single loci conferring resistance against the bacterial pathogen *Pseudomonas syringae* pv. *tomato* encoded by the *Pto* locus (Martin *et al.* 1993; Salmeron *et al.* 1996), against the fungus *Fusarium oxysporum* (Ori *et al.* 1997) and against two races of the fungus *Cladosporium fulvum* encoded by the *Cf-9* and *Cf-2* loci (Jones *et al.* 1994; Dixon *et al.* 1996), have been reported in tomato. The cloning of plant disease resistance genes along with the isolation of the avirulence factors from the corresponding pathogens, provides a solid base for unraveling the mechanism of host resistance.

Resistance to three species of root-knot nematodes from the genus *Meloidogyne*, *M. incognita, M. javanica* and *M. arenaria*, has been introgressed in the cultivated tomato from the wild species *L. peruvianum* and assigned to a single locus, *Mi-1*, on chromosome 6 (Smith 1944; Gilbert 1958). The presence of the *Mi-1* allele ensures the expression of a hypersensitive reaction in the roots invaded by root-knot nematodes at temperatures below 28°C. The *Mi-1*-mediated resistance has been widely exploited in breeding programs for more than 50 years, thus, showing a remarkable durability. This has resulted in a collection of tomato lines that carry, to various extents, a *L. peruvianum* introgression harboring *Mi-1* (Messeguer *et al.* 1991; Ho *et al.* 1992). The abundance of polymorphisms between *L esculentum* and *L. peruvianum* has been used to identify molecular markers diagnostic for *Mi-1*, including the isozyme marker acid phosphatase-1^1 (*Aps-*1^1)(Rick and Fobes, 1974), RFLPs (Klein-Lankhorst *et al.* 1991a; Ho *et al.* 1992), RAPDs (Klein-Lankhorst *et al.* 1991b), a SCAR (Williamson *et al.* 1994) and AFLPs (Keygene, Wageningen, in preparation), thereby delimiting the *Mi-1* locus to a 650 kb region from *L. peruvianum* DNA (Fig. 1). The same 650kb segment has been found recently to carry the *Meu1* locus that confers resistance to

potato aphid (*Macrosiphum euphorbiae*) (Kaloshian *et al.* 1995; Williamson *et al.*, unpublished). Two *Cladosporium fulvum* resistance loci, *Cf-2* and *Cf-5*, are at most 300kb above the *Mi-1/Meu1* region (Dixon *et al.* 1995; Dixon *et al.* 1996). Such clustering of functionally related genes suggests that disease resistance genes may have originated by duplication of an ancient resistance gene and have evolved to different specificities, yet retaining some sequence homology (Pryor 1987). Indeed, the recently isolated *Cf-2* locus comprises two independently functional *Cf-2* genes that are almost identical in sequence (Dixon *et al.* 1996). On the other hand, it is not clear if the linkage of alleles of the same gene or linkage of distinct genes that provide resistance to taxonomically unrelated pathogens, has any significance for their expression, regulation and function.

Here, we have studied a tomato genotype OT745, which has lost spontaneously the *Mi-1*-and *Meu-1*-encoded resistances. This genotype has been identified accidentally during a breeding program in which a nematode susceptible tomato line was crossed to a line that carried the disease resistance genes I_2, *Tm2a* and *Mi-1* and *Meu1*, the latter two loci being present in a large *L. peruvianum* introgression segment that covers the short arm and the centromere proximal region of the long arm including the $Aps\text{-}1^1$ allele. *Tm2a* confers resistance to tobacco mosaic virus and maps on chromosome 9 (Young *et al.* 1988), while the I_2 gene against *Fusarium oxysporum* is located on chromosome 11 (Sarfatti *et al.* 1989). In the course of the breeding program the hybrid and its successive selfed progenies were all tested for nematode resistance on the basis of the isozyme acid phosphatase-1^1 ($Aps\text{-}1^1$) allele, which is tightly linked to *Mi-1*. The *L. peruvianum* allele $Aps\text{-}1^1$ diagnostic for the presence of *Mi-1* (Rick and Fobes, 1974) was found to segregate in the F_1 -F_3 generations, but appeared homozygous in the successive five (F_4 -F_8) generations. Disease resistance bioassays revealed that, although F_8 individuals had retained the *L. peruvianum* allele for *Aps-1*, they had lost nematode and potato aphid resistance but not the *Tm2a* and I_2 resistances. The loss of the *Mi-1* resistance was traced back to an event in the F_4 generation. The F_8 individuals are referred to as the OT745 genotype and are regarded as valuable genetic material to study the relationship between the *Mi-1* and *Meu1* genes and eventually to identify their sequence or control elements of their expression.

Materials & methods

Plant material

Seeds from OT745 were kindly provided by Drs. J. Lambalk from the Dutch seed company Enza Zaden, Enkhuizen. Tomato lines VFNT Cherry and Moneymaker were used as controls in this study.

Mi-1 and Meu-1 disease resistance tests

Root-knot nematode resistance assays were performed on one month-old plants as described by Liharska *et al.* (1997). Tests for aphid resistance were carried out in Davis, California, as described before by Kaloshian *et al.* (1995).

DNA isolation and digestion

DNA for Southern analysis was isolated from leaves and roots as previously described (van der Beek *et al.* 1992). High molecular weight (HMW) plant DNA was prepared from nuclei isolated from few-weeks old leaves following a protocol of Liu and Whittier (1994) with the modification that the nuclei were filtered through a series of nylon meshes of 170, 120, 50 and finally 20mm and pelleted at 3000 rpm. Agarose plugs rather than microbeads were used to embed the nuclei since HMW DNA in microbeads was found to be unstable upon a few weeks of storage in the TE buffer.

YAC 1256 (Fig. 1) spanning the *Mi-1* gene originated from a YAC library made from a VFNT Cherry line (Keygene, in preparation). Yeast cells containing YAC 1256 were kindly provided by Keygene, Wageningen. Yeast HMW was isolated as described by van Daelen *et al.* (1989).

Restriction enzyme digestion of HMW DNA was carried out applying three times 20 units of enzyme per plug in a total of 6 hours incubation.

Pulsed Field Gel Electrophoresis (PFGE)

Plant and YAC digests of HMW DNA were separated on 1% (w/v) agarose gels in 0.5x TBE at 10°C using a CHEF apparatus (CHEF DR II, BioRad, Hercules, USA). The YAC digestion products were separated at 160V over 24h with switching times ramping from 1sec. to 5sec. Conditions for tomato DNA were varied as specified in Table 2. Gels were stained with ethidium bromide, blotted on Gene Screen Plus membrane and hybridized at the conditions recommended by Dupont N.E.N. (Boston, USA). PFGE marker I λ-ladder from Boehringer, Mannheim was used as a molecular size marker in all experiments.

Cloning and sequencing of cosmids from the Mi-1 region

Cosmids derived from YAC 1256 were obtained from Keygene as HindIII x EcoRI or HindIII x EcoRI x PstI digests. HindIII x EcoRI fragments were recovered by directional cloning in plasmid vector pUC18(Sambrook *et al.* 1989). Preparation of the vector DNA, ligation of fragments and subsequent electrotransformation of DH5α cell was according to Sambrook *et al.* (1989). The cloned cosmid HindIII x EcoRI fragments were isolated by direct PCR using vector-specific (M13F and M13R) primers on freshly-grown recombinant colonies.

Cloned HindIII x EcoRI fragments were sequenced using a TaqDye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, California, USA) and an automatic sequencing apparatus of Applied Biosystems.

PCR conditions.

The obtained sequence information was used to design primers to amplify the Cla I-polymorphic sequence. The following synthetic oligonucleotides were used: 32.5Cla F, 5'-ACA CGA AAC AAA GTG CCA AG-3'; 32.5Cla-R, 5'-CCA CCA CCA AAC AGG AGT GTG-3' in 25-cycles of 30sec. 94°C, 1min. 60°C, 1min. 72°C PCR.

Results

Genetic analysis of the OT745 genotype

Disease resistance tests carried out independently in Wageningen and Davis confirmed that the original F8 plants as found before by Enza Zaden were indeed all susceptible to both root-knot nematodes and potato aphid. Further genetic analysis involving a cross of OT745 to both a susceptible (Moneymaker, *mi-1*) and a resistant tomato line (VFNT Cherry, *Mi-1*) revealed that the OT745 "mutant" phenotype was complemented by the *Mi-1* line but not by the *mi-1* line. All hybrid plants from the cross with VFNT Cherry were resistant, whereas the ones from the Moneymaker cross were susceptible to root knot nematodes. These results imply that OT745 does not contain an active *Mi-1* allele due to a recessive mutation which is located within the *Mi-1* introgression. As the tightly linked *Meu-1* gene was likely to be affected by the same mutation, we refer to the mutation as *lmm* for loss of *Mi-1* and *Meu-1* .

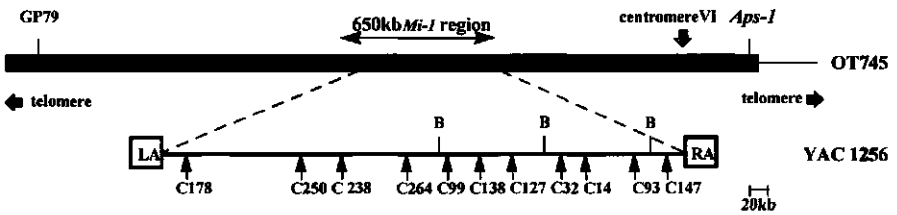

Fig. 1. OT745 line carries an *L. peruvianum* introgression (black bar) that includes markers GP79 and *Aps-1* flanking *Mi-1*. YAC 1256 covers 500kb of the 650kb *Mi-1* region. The arrows are pointing to the approximate (±15kb) position of the cosmids(C) on the physical map of YAC 1256 made with the enzyme BssH II (**B**).

Search for the lmm mutation using conventional RFLPs

On the assumption that the identification of a DNA polymorphisms between OT745 and a parental, nematode resistant line carrying a similar introgression from *L. peruvianum* would point to the *lmm* mutation, the molecular organization of the *Mi-1* region in OT745 was studied using diagnostic molecular markers. VFNT Cherry was chosen as a control *Mi-1* line as this line has been studied, both genetically and molecularly in much detail and carries an introgressed *L. peruvianum* segment of similar size (Fig. 1). In this respect, it is relevant to note here that all present *Mi-1* tomato lines are known to originate from a single hybrid *L. esculentum* x *L. peruvianum* plant, obtained by Smith in 1944 and that no DNA polymorphisms between these lines for the *Mi-1* introgression have been reported so far (see Messeguer *et al.* 1991; Ho *et al.* 1992).

RFLP analysis, using the *Mi-1* -linked markers GP79, LC379 and *Aps-1* (Klein-Lankhorst *et al.* 1991; Ho *et al.* 1992) revealed that OT745 was homozygous for the respective *L. peruvianum* alleles and indistinguishable from VFNT Cherry. Similarly, no differences between OT745 and VFNT Cherry were found for the SCAR marker REX-1 (Williamson *et al.* 1994) and several AFLP markers from the *Mi-1* region (Pieter Vos, personal communication).

Table 1. Cosmid probes used to search for RFLPs on Southerns with 9 enzymes : EcoRI, EcoRV, HindIII, BamHI, DraI, HaeIII, Bgl II, XbaI and TaqI. Polymorphisms between *Mi-1* containing lines (*L. peruvianum*) and *mi-1* lines (*L. esculentum*) were found for most of the 9 enzymes with these probes.

cosmid	fragment no.	size	polymorphisms
178	1	0.7	all
250	5	1.2	EcoRI, EcoRV, DraI, HaeIII, XbaI, TaqI
	47	3.9	EcoRI, EcoRV,DraI, HaeIII, Bgl II, XbaI,Taq I
238	32	1.7	all
264	2	2.0	all
	3	1.5	HindIII, DraI, HaeIII
99	32	3.0	all
138	2	1.0	none
127	2	1.0	EcoRI, EcoRV, BamHI, DraI, Bgl II, XbaI
32	1	2.0	all but HindIII
	5	4.0	EcoRI, EcoRV,DraI, HaeIII, Bgl II, XbaI,TaqI
14	12	1.0	all
93	1	2.4	EcoRI, EcoRV, BamHI, Bgl II, XbaI, TaqI
	3	1.1	EcoRI, EcoRV, HindIII,Bgl II, XbaI, TaqI
147	2	0.8	EcoRI, EcoRV, HindIII,Bgl II, XbaI, TaqI
154	2	0.5	all

Apparently, no major deletions nor recombination events had occurred leading to the nematode- and aphid susceptible phenotype of OT745. Taking into account that the above mentioned markers are dispersed over, the physically very large, *GP79-Aps-1* region (van Daelen *et al.* 1993), these first results suggested that a more detailed, fine mapping was needed to identify the *lmm* mutation. To this end, additional RFLP probes were developed taking advantage of the available YAC- and cosmid- libraries prepared from a *Mi-1* containing tomato genotype (Vos, in preparation). Cosmid clones, derived from a YAC spanning the *Mi-1* region were chosen and EcoR I-Hind III fragments detecting single-copy sequences were selected by screening Southern blots of OT745, VFNT Cherry and Moneymaker (Table 1).

All selected fragments revealed polymorphisms between VFNT Cherry and Moneymaker for most of the nine restriction enzymes applied. However, again, no differences between OT745 and VFNT Cherry were found with any of the newly developed RFLP probes. Yet, as the resolution limit of the conventional Southern analysis is limited to 10-15kb and, on the other hand, the physical distance between the cloned fragments is at least 30kb, a considerable portion of the *Mi-1* region remained unexplored for polymorphisms as detected by this set of RFLPs probes.

Long-range restriction mapping of OT745

Rather than developing more RFLP probes to saturate the entire 650 kb *Mi-1* region, we chose to screen restriction fragments in the range of 10-150 kb as resolved by PFGE for polymorphism employing the present set of probes. Long-range mapping with RFLP probes has already been shown in *Arabidopsis* to reveal more polymorphism than conventional Southern blots (Bancroft *et al.* 1992). Among a wide variety of rare cutting enzymes tested at various PFGE regimes, the enzymes Pac I and Xho I were found most appropriate to generate restriction fragments in the desired range of 10-150 kb. Only one enzyme (Cla I) of the 15 enzymes tested, detected a polymorphisms between OT745 and VFNT Cherry following sequential hybridization with probes 264.2, 127.2, 32.1 and 93.1. In all these cases, an additional Cla I site in VFNT Cherry or loss of a Cla I site in OT745 must be the reason for the observed differences between the two genotypes. Probes 93.1 and 32.1, which are physically linked, resulted in the same polymorphic pattern (Fig. 2).

Identification and isolation of the polymorphic Cla I site

In order to locate the polymorphic Cla I site within the corresponding YAC, a physical map of the entire YAC 1256 was constructed using both single and double digests of the enzymes Cla I, Apa I, Sal I, BssH II and Mlu I. In the tomato genome most of the recognition sites of these enzymes are methylated and not restricted (Messeguer *et al.* 1991).

Hybridizations with probes 93.1, 32.1 and 127.2 of PFGE blots of Cla I-Apa I digests of genomic DNA(Fig. 2, lanes 5-8) indicated the position the polymorphic Cla I site on the physical map, namely on cosmid 32. Further hybridizations of the same blots with probe 32.5 pointed out that the polymorphic site is contained within this 4kb Eco RI-Hind III fragment of cosmid 32. The restriction map of fragment 32.5 (Fig. 3B) as well as its sequence showed indeed the presence of a Cla I recognition site. Since the cosmids contain tomato DNA from the VFNT Cherry line, the presence of an intact Cla I site was in accordance with the original PFGE polymorphism found between this line and OT745 (Fig. 2).

Table 2. List of restriction enzymes used to digest HMW tomato genomic DNA and the conditions to separate the restricted fragments. # **A**: 25-40 sec, 24 h, 200 V; **B**: 2-25 sec, 24 h, 200 V; **C**: 1-15 sec, 24 h, 185 V; **D**: conventional Southern gel.

enzyme	Recognition site	Size of detectable fragments (kb)	Separation conditions#
Apa I	GGGCC/C	20-300	A,B
Bam H I	G/GATCC	<10	D
Bgl II	A/GATCT	<50	D
Cla I	AT/CGAT	10-300	A,B
Dra I	TTT/AAA	<10	D
Eag I	C/GGCCG	10-500	A,C
Eco RI	G/AATTC	<10	D
Eco RV	GAT/ATC	<10	D
Hae III	GG/CC	<10	D
Hind III	A/AGCTT	<10	D
NaeI	GCC/GGC	25-300	A,B
Nde I	A/CGCGT	<50	D,B,C
Sfi I	GGCCN$_4$NGGCC	10-500	A
Pac I	TTAAT/TAA	10-80	C
Pst I	CTGCA/G	40-600	A,B,C
Sal I	G/TCGAC	10-500	A
Sna B I	TAC/GTA	25-300	A,B
Sst I	GAGCTC	1-100	B,C
Sst II	CCGCGG	30-600	A
Stu I	AGG/CCT	5-100	B,C
Taq I	T/CGA	<10	D
Xba I	T/CTAGA	<50	D,B,C
Xho I	C/TCGAG	10-200	B,C

PCR primers were designed to amplify a part of the 32.5 clone that comprises the polymorphic Cla I site (Fig. 3B). A PCR product of 773bp was amplified on VFNT Cherry, OT745 and Moneymaker genomic DNA (Fig. 4). Apparently, there are no major sequence differences such as deletions or other chromosomal rearrangements between these genotypes. Subsequent treatment of the three PCR products with Cla I gave rise in all three cases to two fragments of the expected size as predicted from the sequence of the 32.5 fragment (Fig. 4A). This result indicated that there are no sequence differences between the three genotypes within the Cla I site.

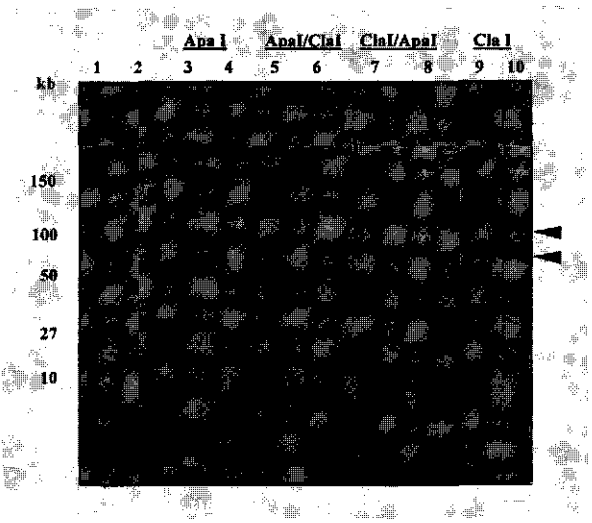

Fig.2 Hybridization pattern of probe 93.1 on a PFGE blot containing OT745 leaves(lanes1,3,5,7,9)and VFNT Cherry leaves(lanes2,4,6,8,10) DNA : undigested (1,2); Apa I (3,4); Cla I (9, 10) and Apa I/Cla I double digest (5-8). The arrows indicate the polymorphic Cla I fragments.

However, when genomic DNA was first digested with Cla I and then used as template for PCR, a quantitative difference appeared between the product of VFNT Cherry and the products directed by OT745 and Moneymaker (Fig. 4B). Reproducibly, a tiny amount of PCR product was made on the VFNT Cherry template after cleavage with Cla I, whereas the fragments made from OT745 and Moneymaker were of the same intensity as were the PCR products made on undigested DNA. The quantitative differences were found when using template DNAs isolated from either leaves and nematode-infected roots (data not shown). From these results, we conclude that changes in the methylation status of the Cla I recognition site rather then base pair changes brought about the Cla I polymorphism differentiating VFNT Cherry and OT745. Cla I is a methylation sensitive enzyme that will not cleave if the cytosine from its recognition site ATC*/GAT is methylated. Thus, in both OT745 genotype and Moneymaker this Cla I site must be fully methylated whereas in VFNT Cherry, the Cla I site is not or partly methylated.

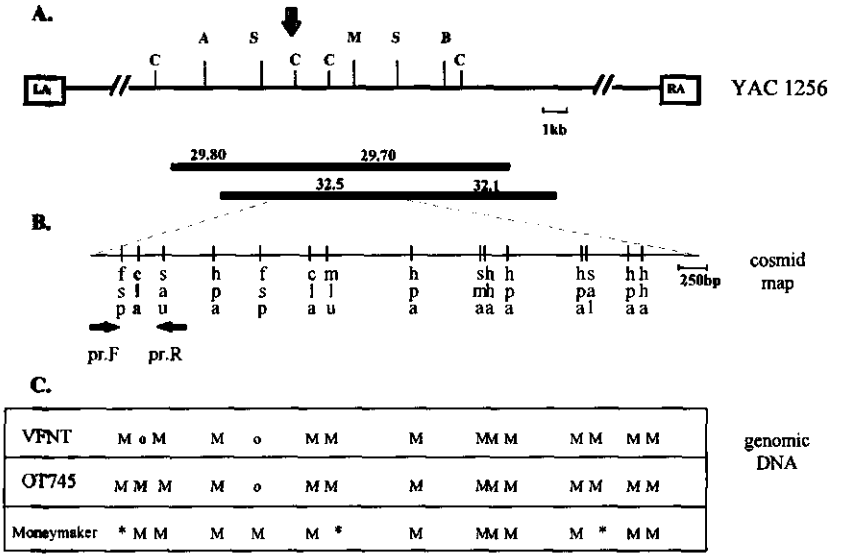

Fig. 3 **A.** Part of the physical map of YAC1256, around probe 32.1. The polymorphic Cla I site is pointed with an arrow. 29.80 and 29.70 are EcoRI-HindIII fragments from cosmid 29 which overlaps C32. The recognition sites of the enzymes A= Apa I; B= BssH II; C = ClaI; M = MluI; S = Sal I are indicated. **B.** Detailed restriction enzyme map of 32.1 and 29.70 fragments. **C.** Sequence analysis and methylation status of genomic DNA for the same region in the three studied genotypes. M=methylated; o = not methylated; *= recognition site not present.

We searched for more differences between OT745, VFNT Cherry and Moneymaker in a region of 6kb that includes the fragment 32.5 and the right-hand neighboring fragment 29.70 (Fig. 3). Genomic DNA from the three genotypes was digested with each of the methylation sensitive enzymes deduced from the sequence information from this 6kb region (Fig. 3B) and subjected to PCR with primers comprising their recognition site(s) (data not shown). No additional methylation differences between OT745 and VFNT Cherry in the 6kb region were identified in this way. For the *L. peruvianum* (VFNT Cherry) and *L. esculentum* (Moneymaker) alleles only one additional site (Fsp I), except for the original Cla I, was found to be differentially methylated (Fig. 3C) .

Fig. 4. PCR products with primers 32.5F and 32.5R on Moneymaker (1,4,7), VFNT Cherry (2,5,8) and OT745(3,6,9) genomic DNA. The PCR products in lanes 4-6 were digested with Cla I. In lanes 7-9 the template DNAs were digested with Cla I prior the PCR.

Discussion

In this report we describe the search for the putative mutation *lmm* in a tomato genotype OT745 that has lost resistance to two different pathogens, root knot nematodes (*Mi-1*) and potato aphid (*Meu1*). The spontaneous origin of *lmm* in a hybrid, heterozygous for the *L. peruvianum* introgression carrying the *Mi-1* and *Meu1* genes, could be the consequence of either, a rare recombination or gene conversion event, or of a chromosomal rearrangement. Considering that *lmm* does not affect the resistance genes *Tm2a* and I_2 placed on other chromosomes, the *lmm* mutation was likely to be located in close vicinity if not within the *Mi-1* and *Meu1* genes, rather then elsewhere in the genome. The genetic data, reported here support this assumption, indicating that *lmm* is contained within the *Mi-1* introgression.

The search with random RFLP probes from the *Mi-1* region for the *lmm* mutation did not result in the identification of any polymorphism between OT745 and the *Mi-1* line VFNT Cherry, suggesting that no gross rearrangement had occurred that could have given rise to the *lmm* mutation. Using long-range mapping with RFLP probes, only one polymorphism, for a Cla I site, was found between *lmm* and VFNT Cherry upon testing 15 different restriction enzymes and four RFLP probes. Although the probes covered the 650 *Mi-1* region at the resolution of PFGE, it was possible that other polymorphisms within this region have remained uncovered. Whether the Cla I polymorphism is the cause of the OT745 phenotype and thus represents the *lmm* mutation, is unknown, but as yet it is the only molecular difference found so far between OT745 and VFNT Cherry.

We were able to locate the polymorphic Cla I site close to the right end of the 650kb *Mi-1-Meu1* region as a hypermethylated site in the susceptible (OT745 and Moneymaker) genotypes. The present methylation does not seem to be tissue specific since the same polymorphism was seen in DNA isolated from both leaves and roots(results are not shown). This finding further supports the association between the Cla I polymorphism and the *lmm* mutation, since *lmm* is affecting the expression of resistance to root knot nematodes in roots and to potato aphid in leaves.

At this stage of the characterization of the OT745 mutant it is unclear as to which role the differentially methylated Cla I site can play in the loss of resistance in OT745 and how this mutation originated and is maintained. DNA methylation is a well known mechanism in both plants and animals to regulate gene activity during development by stably altering the local structure of the gene. Methylation-dependent repression of transcription of *Mi-1* and *Meu1* could have occurred in OT745 and maintained through the generations. Since no other putative methylation sites around the Cla I site were found to be differentially methylated in OT745, it seems that rather a site-specific methylation than an overall elevated level of methylation is the cause for the *lmm* phenotype. Therefore our current interest is to localize the Cla I site relative to the two resistance genes and to further characterize the Cla I region.

From the recent progress made in the molecular mapping of *Mi-1* and *Meu1*, it becomes evident that they do not contain but flank the polymorphic Cla I site (Chapter 3; Williamson, unpublished). Moreover, sequence analysis of the Cla I region revealed that the Cla I site is a part of an ORF with homology to various human and mice proteins with various functions. Further molecular cloning and expression studies with the "Cla I gene" are in progress in our lab.

Acknowledgements

We thank Drs. Joep Lambalk from EnzaZaden for providing seeds of OT745; Keygene B.V. Wageningen for the YAC 1256 and the cosmid clones digests and for sharing unpublished results; John Boudeaux for providing sequence information of the *Mi-1* region. We also thank Maarten Koornneef for the useful discussions and for critical reading of the manuscript.

Chapter 6

GENERAL DISCUSSION

On the isolation of disease resistance genes in plants via positional cloning

In the early 1990's the technique of positional (or map-based) gene cloning offered a great challenge for plant geneticists. Isolating genes based solely on their position in the genome required a well defined genetic map, tightly linked molecular markers such as RFLPs, genomic libraries with large inserts (a Yeast Artificial Chromosome, YAC, library was "a must") and an efficient protocol for *Agrobacterium*-mediated transformation of the respective plant species needed to perform complementation analysis. Plant disease resistance genes seemed to be good candidates to be isolated via this approach since they are identified genetically, their products were unknown and they play a key role in defining different resistant specificities in the host defense response.

Change in the paradigm for positional cloning

The strategy of map based cloning as applied originally involved a "walk" from the flanking markers towards the gene of interest using overlapping YACs until a clone carrying the putative gene was identified. This YAC clone could either be used for direct screening of a cDNA or cosmid library, or, directly, for complementation analysis, although the later has not yet been made technically possible in plants. However, working with YAC contigs proved to be troublesome quite soon. Along with the difficulty of handling such large molecules, rearrangements within the YACs and the occurrence of chimerical clones hurdled the successful application of map-based cloning.

Recent developments in molecular marker technology have significant impact on the way this approach is currently being applied in plants. With the appearance of RAPD and AFLP markers it became possible to gain access to any part of a plant genome and thus to obtain a sufficient number of markers linked to a target gene. The isolated markers can be ordered relative to the target gene using high resolution genetic mapping. The markers closest to the gene are selected to identify a YAC or a few cosmid clones likely to carry the target gene sequence. In this way, "landing" at the gene rather then "walking" to is performed as a major step in the positional cloning approach (Tanksley *et al.* 1995). Chromosome landing at a genomic clone with the putative gene bypasses one of the major obstacles associated with the aforementioned approach, namely walking through repetitive sequences that are a major constituent of plant genomes.

The tomato gene *Pto* that confers resistance against the bacterial pathogen *Pseudomonas syringae* pv. tomato became the first resistance gene to be isolated via chromosomal landing and was published in 1993 by Martin *et al.*. Since then, a variety of resistance genes from several plant species have been isolated by means of chromosome landing (Table 1). Alternatively, transposon tagging has been exploited successfully in some

cases (Table 1). At present one should consider the accumulated knowledge and experience in this direction in making a choice between different cloning approaches. In considering the constitutive expression of resistance genes in both resistant and susceptible genotypes, differential cDNA library screening, an approach originally anticipated, would clearly be fruitless. On the other hand, as plant disease resistance genes are often found as a cluster of single genes with multiple alleles or as complex loci of tightly linked genes, preparing for chromosome landing requires genetic mapping at very high resolution to allow the identification of the particular member of an array of related genes responsible for the particular resistance specificity. Clustering of resistance gene homologues with a similar or related function in plant defense response can also complicate the outcome of the complementation analysis as a final step of positional cloning. For example, a gene tagging approach would have never been successful in the case of the tomato *Cf-2* resistant locus which comprises two functional homologues.

Chromosome landing at the *Mi-1* gene

The work described in this thesis is a part of the joint effort of three laboratories to isolate the root-knot nematode resistance gene *Mi-1* in tomato: the Department of Molecular Biology in Wageningen (Dr. Pim Zabel), the Department of Nematology, UCD, Davis, USA (Dr. Valerie Williamson) and Keygene N.V., Wageningen. At the start of this collaboration, two tightly linked markers *Aps-1* and *GP79* (1cM) flanking *Mi-1* and their corresponding YAC clones were available. From the lack of overlap between these YAC clones and between the long-range physical maps of *Aps-1* and *GP79*, it was obvious that these flanking markers were more than 1,2Mb apart (Van Daelen 1995). At the same time a tomato line, Motelle, was found that carries approximately 650kb of *L. peruvianum* introgression along with *Mi-1*. This line seemed very suitable to search for additional molecular markers closely linked to *Mi-1* and for high-resolution mapping of the gene.

Markers

In our laboratory at first we conducted a screen of a set of nearly isogenic lines such as Motelle and Moneymaker, and 83M/R and 83M/S with 120 single OPERON primers and pairs of them to amplify RAPD markers tightly linked to *Mi-1* (Klein-Lankhorst *et al.* 1991b; Liharska, unpublished). Thus, a number of RAPD bands that differed between the abovementioned NILs was found, however further characterization of these RAPD markers led to the conclusion that none of them was diagnostic for the *Mi-1* introgression of Motelle (Liharska, unpublished). In a similar study involving an additional 160 OPERON primers,

Williamson *et al.* (1994) isolated one RAPD marker, which showed tight linkage to *Mi-1* within the introgression of Motelle line. This marker was converted to a SCAR, REX-1, that proved to be highly useful in the further genetic analysis of the *Mi-1* locus. Thus, although facile and relatively rapid, in our experience the RAPD technique did not justify any further efforts to isolate a large number of markers for high-resolution mapping. In a relatively short time more than 20 AFLP markers for the Motelle *Mi-1* region were identified using NILs (Vos *et al.* in preparation). These AFLPs served to pull out a new set of YACs covering the *L. peruvianum* introgression and, thus, to establish a physical map of the *Mi-1* region. Most often both RAPDs and AFLPs appeared as dominant markers which limited their application in genetic mapping.

One RFLP marker (LC379), a member of a multigene family, was found diagnostic for the Motelle region (Ho *et al.* 1992) and was employed together with the corresponding polymorphic 1.1kb EcoRI genomic fragment in our studies.(Chapter 4). In later stages we applied random genomic clones in cosmids to generate probes for RFLP mapping (Chapters 4 and 5). These RFLP markers were very informative for the fine mapping of recombinants and the analysis of the spontaneous mutation in the OT745 line.

High-resolution mapping

The most serious problem associated with chromosome landing remains the resolution that can be achieved with genetic mapping. It was clear from previous studies that in the *Mi-1* region recombination frequency is considerably lower than the the genome average (Messeguer *et al.* 1991; Ho *et al.* 1992), as a result of which large segregating populations would be needed for identifying useful recombinant individuals. To deal with a large number of plants, we employed morphological and PCR markers flanking *Mi-1* to pre-select among F_2 plants those with crossovers in the *Mi-1* region (Chapters 2 and 4). Tomato resistant lines with different sized *L. peruvianum* introgressions along with *Mi-1* were used to create the segregating populations. On basis of the distribution of the recombination frequencies in these crosses as determined by molecular markers, it became apparent that recombination was suppressed within the introgressions of these lines (Chapter 2). Consequently, for the purpose of high-resolution mapping, interspecific crosses rather than backcrossing of introgression lines had to be chosen. To this end, crosses within *L. peruvianum* accessions, the source of *Mi-1*, were made, resulting in 20-fold higher recombination than in the *L. esculentum* crosses containing *Mi-1*. *L. peruvianum* is a heterogeneous species, which offers *Mi*-susceptible accessions and sufficient DNA polymorphisms among different accessions. On the other hand, the presence of genes in the wild species that interfere with *Mi-1* resistance may cause

serious problems in interpreting the results of the nematode bioassay. Also, the application of molecular markers polymorphic for *L. esculentum /L. peruvianum* was not always possible in crosses involving *L. peruvianum* accessions, because of a change in the pattern of these markers. Nevertheless, we were able to combine the data from respective *L. esculentum* and *L. peruvianum* crosses thereby narrowing down the *Mi-1* locus to a segment of 65kb.

With the view of making a high-resolution map we also exploited irradiation-induced deletions made in the *Mi-1*-resistant line VFNT Cherry (Chapter 5). Screening for pseudo-dominants at four loci, *tl, yv ,c* and *Mi-1* revealed that the induced deletions were mainly large and terminal with most of the breakpoints within the pericentromeric heterochromatin. Unfortunately, no useful deletions regarding the *Mi-1* locus were obtained, thus limiting the application of this particular approach that was aimed at resolving a cluster of molecular markers around *Mi-1*.

Study of a spontaneous mutant

In the course of our study we identified a mutant that affected the resistance gene specificities to both root-knot nematodes (*Mi-1*) and to potato aphids (*Meu*1) and embarked on a search for the putative mutation *lmm* (Chapter 5). Originally, we considered *lmm* as a part of the *Mi-1* gene and used cosmid-derived RFLP probes to map the mutation. More recently, however, evidence has been obtained from recombinant analysis showing that nematode (*Mi-1*) and aphid (*Meu1*) resistance correspond to separate loci (Williamson, personal communication), a finding that makes this mutant even more valuable and suggests a role of the putative *lmm* gene as a common component for transduction of distinct signals in the plant defense mechanism.

Identification of the Mi-1 gene

Narrowing down the *Mi-1* locus to a 65 kb *L peruvianum* segment as a result of various genetic analysis permitted complementation analysis with some selected cosmid clones and led to the identification of a genomic clone capable of complementing a susceptible genotype (P.Vos *et al.*, unpublished). From the preliminary sequence data it seems that the root-knot nematode resistance gene in tomato is similar to other resistance genes identified so far (Table 1). It contains 13 leucine rich repeats (LRR) and a nucleotide binding site (NBS) domain and, thus, falls in the first class of resistance genes (Salmeron *et al.* 1996). Of particular interest is the homology found with the beet cyst nematode resistance gene Hs1 $^{pro-1}$ (Cai *et al.* 1997). Although the *Mi-1* and Hs1 $^{pro-1}$ genes confer resistance to similar pathogens, they trigger different responses concerning the timing and tissue localization of

the HR. As for the other identified resistance genes it is still unclear as to how this structural similarity between different resistance genes can explain their intrinsic properties of receptors.

It still remains to be seen what the relationship is between the *Mi-1* and the *Meu1* gene. *Mi-1* belongs to a multigene family of 12 -13 members, clustered within the Motelle region. It is likely that one of the homologues corresponds to the *Meu1* gene. Complementation analysis is underway to test this assumption (Williamson *et al.*, personal communication).

Molecular cloning of candidate disease resistance genes

Most of the disease resistance genes isolated so far share structural homology, although they carry unique sequences at the DNA level (Table 1). Except for the *Pto* gene, which is a protein kinase, they all contain a LRR motif and thus belong to the large group of LRR-containing proteins, considered to play a role in protein-protein interactions by accommodating a unique structure (Kobe and Deisenhofer 1994). The LRR domain of the resistance genes is not very well conserved as compared to the consensus sequence of the LRR superfamily and probably serves somehow in their receptor-like function and downstream interaction. Some of the isolated resistance genes (group 1, Salmeron *et al.* 1996) contain a NBS domain (or P-loop) that is found in many ATP-and GTP-binding proteins but the function of which is not clear yet in resistance genes. The structural similarities between resistance genes isolated from different plant species with specificity for a wide variety of viral, bacterial, and fungal pathogens, offer the opportunity to isolate similar sequences using PCR.

Degenerated primers designed to conserved domains of NBS have been used to amplify NBS-encoding sequences from potato and soybean (Leister *et al.* 1995; Kanazin *et al.* 1995;Yu *et al.* 1995). Some of the isolated sequences contain the characteristic LRR motifs and map to known resistance genes. However, since not all resistance genes contain an NBS domain not all resistance like gene (RLG) sequences can be isolated by means of such primers. Concerved sequences within the LRR, if found, would be more useful to amplify RLG homologues.

At present, combining this PCR approach with the traditional positional cloning and gene tagging approaches should allow one to gain quick access to novel resistance genes in any plant genome. Also, from the accumulating sequence information from model plants such as *Arabidopsis* and tomato one can deduce RLG sequences on the basis of homology. Of course

Table 1. Cloned plant disease resistance genes*

R gene	Plant	Pathogen	Avr gene	Cloning method	Structure	Predicted localization**	Reference
Pto	Tomato	*Pseudomonas syringae* pv. tomato	*AvrPto*	map-based	Protein kinase	plasma membrane	Martin *et al.* (1993)
Cf-9	Tomato	*Cladosporium fulvum*	*Avr9*	Tn tagging	LRR	extracellular	Jones *et al.* (1994)
Cf-2	Tomato	*C. fulvum* Avr2	*Avr2*	map-based	LRR	extracellular	Dixon *et al.* (1996)
Prf	Tomato	*P. syringae* tomatoAvrPto	*AvrPto*	map-based	LRR,NBS, LZ	plasma membrane	Salmeron *et al.* (1996)
I2C-1	Tomato	*Fusarium oxysporum*	?	map-based	LRR,NBS	cytoplasmic	Ori *et al.* (1997)
RPS2	*Arabidopsis*	*P. syringae* tomato	*avrRpt2*	map-based	LRR,NBS, LZ	cytoplasmic	Bent *et al.* (1994); Mindrinos *et al.*(1994)
RPM1	*Arabidopsis*	*P. syringae*pv *maculicola*	*avr RPM1*	map-based	LRR,NBS, LZ	cytoplasmic	Grant *et al.* (1995)
RPP5	*Arabidopsis*	*Peronospora parasitica*	?	map-based	LRR,NBS	cytoplasmic	Parker *et al.* (1997)
N	Tobacco	Tobacco mosaic virus	?	Tn tagging	LRR,NBS	cytoplasmic	Whitham *et al.* (1994)
L6	Flax	*Melampsora lini*	?	Tn tagging	LRR,NBS	cytoplasmic	Lawrence *et al.* (1995)
M	Flax	*Melampsora lini*	*A-M Avr*	Tn tagging	LRR, NBS	cytoplasmic	Anderson *et al.* (1997)
Hs1pro-1	Sugar beet	*Heterodera schachtii*	?	map-based	LRR	plasma membrane	Cai *et al.* (1997)
Xa21	Rice	*Xanthomonas campestris* pv *orizae*	?	map-based	Protein kinase , LRR	extracellular	Song *et al.* (1995)

*genes involved in gene-for-gene plant-pathogen interactions; LRR: leuine-rich repeat; NBS: nucleotide binding site; LZ: leucine zipper;

* * based on comparative sequence analysis.

they might be host resistance genes that are unique in sequence and such candidate gene strategy will not be successful.

Criteria to evaluate sequences isolated as disease resistance genes

While isolating resistance genes or RLG sequences via different approaches the following criteria should be considered (Tanksley *et al.* 1995; Michelmore 1996).

The isolated sequences must co-segregate with the phenotype in the course of the high-resolution mapping. Although probably many more resistance genes will be found equally expressed in the resistant and the susceptible phenotypes, as shown for the *Pto* gene (Jia *et al.* 1997), in some cases the expected expression pattern can serve as a criterion to select for a certain sequence. For example, a cDNA candidate for the beet Hs1 $^{pro-1}$ gene was chosen for further complementation analysis by Cai *et al.* (1997) because it hybridized to DNA only from resistant Hs1 $^{pro-1}$ addition lines. The identified structural homology of a candidate resistant gene sequence to other resistant genes can encourage the further analysis of this clone, but it will not be sufficient to claim the isolation of a novel gene. Also, a sequence that complements the susceptible phenotype or an antisense construct of cDNA that restores susceptibility of a resistant line should be further analyzed to cover some of the other criteria for disease resistance genes. Complementation of susceptible genotypes with RLG-homologues might result in a partial resistant phenotype or even complete resistance and can lead to wrong conclusions. Additional prove can be gained from isolating alleles of the putative gene that are altered in the mutant lines.

In each individual case, different approaches for gene isolation can prove successful, however it is important to apply more than one criteria to evaluate the putative disease resistance genes.

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Samenvatting

Het doel van het onderzoek dat in dit proefschrift beschreven wordt, was de isolatie en karakterisering van het tomaat locus *Mi*-1, dat resistentie verleent tegen plantpathogene wortelknobbelaaltjes van het geslacht *Meloidogyne*, die schade veroorzaken bij een groot aantal gewassen. Resistentie van een plant tegen een ziekteverwekker houdt in dat de plant reageert op infectie met een afweerreactie, die verdere aantasting voorkomt. Bij het tot stand komen van de afweerreactie spelen de resistentiegenen een sleutelrol. Het leek zeer interessant om het *Mi*-1 locus te isoleren en te onderzoeken welk product door dit gen gecodeerd wordt. Op die manier kan inzicht verkregen worden in het mechanisme van de resistentie en de specificiteit van de interactie van wortelknobbelaaltjes en de gastheerplant. Bovendien zou dan onderzocht kunnen worden of overdracht van het Mi-1 gen naar gevoelige planten in die planten ook resitentie tegen wordenknobbellaaltjes bewerkstelligt.

De isolatie van een gen vereist een goed gedefinieerde genetische kaart met een groot aantal moleculaire merkers zoals RFLP's, die nauw gekoppeld zijn aan het fenotype van het gen dat geïsoleerd moet worden. Voorts zijn genomische banken nodig met grote inserties, b.v. een YAC (Yeast Artificial Chromosome) bank, terwijl voor de functionele identificatie van gekloneerde genen via complementatie analyse, een efficiënte methode om planten te transformeren beschikbaar moet zijn. Aan het begin van het onderzoek waren de benodigde technieken voor de tomaat in principe beschikbaar en leek de weg geëffend te zijn om de isolatie van een resistentie gen zoals *Mi*-1 te ondernemen via positionele klonering.

Het *Mi*-1 resistentie locus is afkomstig van de wilde tomatensoort *Lycopersicon peruvianum* en is in de cultuurtomaat *L. esculentum* ingekruist. Het komt nu voor als een introgressie in verschillende nematode resistente lijnen van *L. esculentum.* In *hoofdstuk 1* wordt beschreven welke fenotypische eigenschappen het *Mi*-1 locus aan tomatenplanten geeft en wordt het voorkomen van andere genen voor resistentie tegen wortelknobbelaaltjes in *Lycopersicon* soorten besproken. In dit hoofdstuk wordt ook kort weergegeven wat, bij het begin van het onderzoek, de stand van zaken was van de moleculaire genetica van het *Mi*-1 locus, die de basis moet vormen voor de positionele klonering van *Mi*-1.

Het *Mi*-1 locus ligt op de korte arm van chromosoom 6 van de tomaat. Om kloneren mogelijk te maken is het noodzakelijk om een zo klein mogelijk gebied van het chromosoom af te bakenen waarop het *Mi*-1 locus ligt en zoveel mogelijk fenotypische en moleculaire merkers in dit gebied in kaart te brengen. Daartoe werden een groot aantal recombinanten geanalyseerd. Het bleek dat de recombinatie van *L. esculentum* in het introgressiegebied met

het *Mi*-locus sterk onderdrukt was. Die onderdrukking bleek het gevolg te zijn van de soortvreemde afkomst van het chromosomale DNA met het *Mi*-locus. Het *Mi*-locus ligt bovendien dichtbij het centromeer van chromosoom 6 en die positie kan extra bijdragen aan de onderdrukking van de recombinatie in het gebied dat in kaart gebracht moest worden. Daardoor was het niet mogelijk om het gebied waarin *Mi* ligt nauwkeurig af te bakenen (*hoofdstuk* 2).

Als een alternatieve methode om recombinanten in kaart te brengen zijn straling-geïnduceerde deletiemutanten gebruikt om te proberen met behulp daarvan een verfijnde kaart van moleculaire merkers van het *Mi*-gebied te verkrijgen (*hoofdstuk* 3). Hoewel de verkregen deletiemutanten bruikbaar waren om het niet-recombineerbare gebied rond het centromeer van chromosoom 6 in kaart te brengen, verschafte deze aanpak toch niet de verwachte en vereiste precisering van de genetische kaart. Dit werd veroorzaakt door de niet-willekeurige distributie van de deletiebreekpunten en het feit dat vooral grote terminale deleties werden verkregen.

In *hoofdstuk* 4 wordt vervolgens beschreven hoe twee elkaar aanvullende strategieën zijn gebruikt om meer recombinanten in het *Mi*-gebied te identificeren en in kaart te brengen. Die aanpak bestond aan de ene kant uit een uitbreiding van het onderzoek naar recombinanten, waarvan in *hoofdstuk* 2 verslag is gedaan, met op PCR gebaseerde merkers en AFLP merkers rond de *Mi*-locus in *L. esculentum*. De andere strategie bestond uit het genereren en analyseren van een segregerende populatie van de wilde tomaat *L. peruvianum* , waaruit het *Mi*-locus afkomstig is. Met PCR-merkers lukte het om in die populatie recombinanten te identificeren met kruisingen dicht bij *Mi*.

Op die manier kon het *Mi*-1 locus begrensd worden tot een gebied van het genoom van minder dan 65 kb. Daarmee was de weg vrijgemaakt om *Mi*-1 te identificeren in klonen van YAC banken en in cosmid klonen.

Vervolgens werd het tomatengenotype *L.esculentum* OT745 bestudeerd dat spontaan het *Mi*-1 locus had verloren en tegelijkertijd ook resistentie tegen aardappelluizen (*Macrosiphum euphorbiae*) was kwijtgeraakt (*hoofdstuk* 5). De genetische en moleculaire gegevens wezen op de aanwezigheid van een mutatie (*Imm1*) in het OT745 genotype, gelocaliseerd binnen de introgressie met *Mi*-1, die gepaard gaat met een hypergemethyleerde ClaI herkenningsequentie buiten de gebieden van de *Mi*-1 en *Meu1* genen.

In *hoofdstuk* 6 wordt besproken welke vooruitgang is geboekt met de gebruikte genetische aanpak bij het bestuderen van het *Mi*-1 locus en welke betekenis dit onderzoek is geweest voor de recente vorderingen met de klonering van *Mi*-1. Het onderzoek weerspiegelt de snelle ontwikkeling van de moleculaire genetische technologie voor de identificatie en karakterisering van genen in planten.

Съдържание на дисертацията

"Генетичен и молесулярен анализ на устойчивостта към коренови нематоди при доматите"

Настоящата работа цели изолирането на доматения локус *Mi-1* за устойчивост към коренови нематоди от семейство *Meloidogyne* . Кореновите нематоди са широко разпространени паразити по голям брой културни растения и нанасят големи икономически загуби на селското стопанство в цял свят. Изучаването на естествената устойчивост към тези и други паразити и изолирането на съответните гени за устойчивост има както практическа стойност, така и се явява необходимост за разбирането на взаимодействието между паразити и гостоприемници.

Устойчивост към едни от най-разпространените коренови нематоди *Meloidogyne incognita* е открита в дивия домат *Lycopersicon peruvianum* и пренесена в културния домат *Lycopersicon esculentum* през 40-те години. Тази устойчивост се определя от един доминантен локус *(Mi-1)* на хромозома 6 от доматения геном и е обект на изследване от няколко лаборатории в света. *Глава 1* от дисертацията описва свойствата на тази устойчивост и наличието на други гени открити в рода *Lycopersicon* за устойчивост към коренови нематоди. Също така в *глава 1* е представена стратегията за клониране на *Mi-1* , основана на прецизното картиране на локуса.

За създаването на детайлна молекулярна карта на *Mi-1* локуса, беше необходимо да се получат голям брой рекомбинантни растения с известен фенотип (устойчиви или чувствителни кам коренови нематоди). За това, няколко популации разпадащи се за *Mi-1* учтойчивост бяха създадени. Във всички случаи, като един от родителите на тези популации беше използвана чувствителна към нематоди доматена линия, носеща няколко класически маркера разположени около *Mi-1* локуса. Другият родител беше устойчива *Mi-1* линия. Във второ поколение на тези кръстоски с помощта на морфологичните маркери бяха отбирани растения с рекомбинация в областта на *Mi-1*. Въпреки големият размер на използваните популации обаче, само малък брой рекомбинантни растения бяха получени. Причина за това е силно намалената рекомбинация в района на *Mi-1* , което може да се обясни с отрицателния ефект на близкоразположената центромера на хромозома 6 както и на самия *Mi-1* локус, произлизащ от див вид домати. Резултатите от тези изследвания са представени в *глава 2*.

Друг начин за подобряване на молекулярната карта на *Mi-1* локуса беше използването на индуцирани мутанти с делеции в самия локус. Въпреки че подобни мутанти бяха получени за хромозома 6 (*глава 3*), ние установихме значителна тенденциозност в разпределението на делециите в тази част от генома. Тази особеност на гама-индуцираните делеции ги прави неудобни за картиране на различни части на генома.

Допълнителни рекомбинантни растения бяха получени от кръстоски между линии див домат *Lycopersicon peruvianum*, единият от които, чувствителен към нематоди, а другият-първоначалният източник на *Mi-1* устойчивост. За отбирането на рекомбинанти в тези популации бяха използвани PCR маркери, заобикалящи *Mi-1*. Трзи рекомбинанти заедно с първоначално намерените в *Lycopersicon esculentum* популации, бяха обект на молекулярен анализ с RFLP и AFLP маркери. Като резултат, *Mi-1* локусът беше локализиран в рамките на 65 kb от доматения геном.

В хода на тези изследвания беше намерена линия домати, която в резултат на спонтанна мутация е загубила устойчивост към коренови нематоди (*Mi-1*) и към картофени листни въшки (*Meu 1*). Генетичният и молекулярен анализ на тази линия OT745 доведе до заключението за наличието в този генотип на мутация (*Imm*) в близост до *Mi-1* и *Mieu1*, която е причинена от диференциално метилиране в мутантния геном (*глава5*).

В глава 6 се обсъжда предприетата стратегия за клониране на *Mi-1* в светлината на натрупаната през последните години информацияза други растителни гени за устойчивост към различни патогени и на клонирането на самия *Mi-1* локус.

List of publications

1991

1. Klein-Lankhorst, R.M., Vermunt, A., Weide, R., Liharska, T.B., and Zabel, P. (1991) Isolation of molecular markers for tomato (*L.esculentum*) using random amplified polymorphic DNA (RAPD), *Theor. Appl. Genet.* 83,108-114.

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11. Kaloshian, I. , Yaghoobi, J., Liharska, T.B., Hontelez, J., Hanson, D., Hogan, P., Jesse, T., Wijbrandi, J., Vos, P. , Zabel, P., and Williamson, V. M. (1997) Genetic and physical localization of the root-knot nematode resistance locus *mi* in tomato, *Mol. Gen. Genet., in press.*

Curriculum vitae

Tsvetana Bojidarova Liharska was born in 1962 in Bulgaria. She studied molecular biology at the University of Sofia, where she obtained her MSc degree in Biochemistry in 1985.

From 1986 to 1989 she worked as a research scientist on various projects at the newly founded Institute of Genetic Engineering nearby Sofia. In 1990 she started a PhD research on molecular markers in barley at the same Institute. In January 1991 she came to work in the group of Dr. Pim Zabel, Department of Molecular Biology, Wageningen Agricultural University within the frames of a "sandwich fellowship" provided by this University on RAPD markers in tomato. From 1992 to 1996 she carried out a PhD research on the molecular isolation of the root knot nematode resistance gene in tomato in the same laboratory under the supervision of Dr. Maarten Koornneef and Dr. Ab van Kammen.

Since January 1997 she works as postdoc at the Department of Genetics, Free University of Amsterdam on a EU-funded project for tagging the tomato genome using homologous recombination. She is married with a daughter and lives in Leiden.