The genetics and mechanisms of resistance to tomato powdery mildew (*Oidium neolycopersici*) in *Lycopersicon* species

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Contens

Chapter 1

General introduction

Tomato

Tomato (*Lycopersicon esculentum* Mill) is a very important vegetable both for the fresh market as well as for the processed food industry. Although cultivated as an annual, tomato grows as a perennial in its original habitat in the Andean region. The original site of domestication of tomato is probably in Mexico (Picken et al. 1985, Taylor 1986).

The cultivated tomato, *Lycopersicon esculentum*, belongs to the genus *Lycopersicon* that contains eight wild species (Taylor 1986). Major hybridization barriers divide the *Lycopersicon* genus into two groups, the "*esculentum* complex" and the "*peruvianum* complex". The "*esculentum* complex" consists of seven species, namely *L. esculentum*, *L. pimpinellifolium*, *L. cheesmanii*, *L. chmielewskii*, *L. parviflorum*, *L. hirsutum* and *L. pennellii*. The "*peruvianum* complex" is comprised of two extremely diverse species, *L. chilense* and *L. peruvianum*. Crosses are difficult between the two groups (Rick 1982), but with various embryo rescue techniques, all of the germplasm in the genus is potentially transferable to the cultivated tomato. Many traits of economic importance, like almost all the major disease resistances, originate from wild *Lycopersicon* species (Rick and Yoder 1988).

Tomato is a favorite model species for classical genetic studies, due to its selfcompatibility, easy crossability to wild species and simple diploid genome with 12 chromosomes. Nowadays, tomato has also become a model plant in molecular genetics and genomics, thanks to its small genome size (about 950 Mb/haploid genome) and the availability of a wide range of high density molecular maps, DNA libraries (EST-expressed sequence tag and BAC-bacteria artificial chromosome) and microarrays. Very recently, the tomato genomic research program of the Dutch CBSG project (Centre for BioSystem Genomics) started sequencing a part of Chromosome 6, zooming on a region known to comprise genomic loci that are involved in fruit quality and defense responses to pathogen attack (http://www.biosystemsgenomics.nl/). Furthermore, the recently initiated International Solanaceae Genome Project (SOL) has chosen tomato as the *Solanaceae* model species for genome sequencing (http://www.sgn.cornell.edu/solanaceae-project/). It is expected that the whole tomato genome will be sequenced in a joint effort of several research groups worldwide.

RFLPs (restriction fragment length polymorphisms) and AFLPs (amplified fragment length polymorphisms) are the most frequently used molecular markers for the construction of

genetic linkage maps of *Lycopersicon* species (Bai et al. 2003, Bonnema et al. 2002, Haanstra et al. 1999b, Tanksley et al. 1992). These detailed genetic linkage maps in *Lycopersicon* allow efficient mapping of any important gene in tomato. Simple PCR markers (e.g. CAPS-cleaved amplified polymorphic sequence, SCAR-sequence characterized amplified region) can be developed from AFLPs and RFLPs and used as diagnostic markers for marker assisted selection (MAS). Comparative genetic mapping within the *Solanaceae* family is possible with locus specific markers such as RFLPs and CAPS, which allow comprehensive examination of the genomic organization of a wide array of interesting genes. EST derived COS markers (conserved ortholog sets) are available and are used as the core set of molecular markers to explore the syntenic relationships between the tomato and *Arabidopsis* genomes.

Tomato powdery mildew: *Oidium neolycopersici*

Oidium neolycopersici is a relative recently identified species of tomato powdery mildew. It was first reported in the Netherlands in 1986 and since then has spread rapidly world-wide (Paternotte 1988). The origin of *O. neolycopersici* is still unclear. The identification of *O. neolycopersici* from Asian herbarium specimens in 1947 implied that the appearance of *O. neolycopersici* is not very recent (Kiss et al. 2001). Why *O. neolycopersici* could spread so rapidly around the world is still unknown.

The lack of a sexual stage makes identification of this pathogen ambiguous. Noordeloos and Loerakker (1989) adopted the name *Oidium lycopersicum* for the European tomato powdery mildew fungus, which appeared to come from Australia. This name was changed to *O. lycopersici* in 1999, in accordance with the international Code of Botanical Literature (Mieslerová and Lebeda 1999). However, the pathogen that caused all the recent outbreaks of tomato powdery mildew outside Australia was recently renamed to *O. neolycopersici* based on morphological features of the fungus and DNA sequences of the internal transcribed spacer regions of the nuclear rRNA genes (Jones et al. 2000, Kiss et al. 2001). The Australian isolates retained the name *O. lycopersici* since they most likely represent the original *O. lycopersicum* (Kiss et al. 2001).

O. neolycopersici can easily be distinguished morphologically from another species of tomato powdery mildew, *Leveillula taurica,* which occurs in subtropical regions. The mycelium of *L. taurica* grows into mesophyll of the leaf and is visible on the down side of the leaf, while *O. neolycopersici* grows mainly on the upper side and does not penetrate into the mesopyhll (Lindhout et al. 1994a).

The host range of *O. neolycopersici* is still debatable. As reviewed by Mieslerová and Lebeda (1999), *O. neolycopersici* is able to infect various representatives of the *Solanaceae* and *Cucurbitaceae*. Huang et al. (2000) reported that tobacco (*Nicotiana tabacum*) is an alternative

host for *O. neolycopersici*. Differences in host range reported so far can be caused by the use of different genotypes, environmental conditions, the definition of susceptibility and contamination, but may also indicate the existence of different *O. neolycopersici* isolates (Whipps et al. 1998, Huang et al. 2000a). Limited information is available on the genetic variation in *O. neolycopersici* for virulence or pathogenicity on tomato as tomato differential series with different resistance genes are lacking. It has been reported that a British isolate has higher and specific pathogenicity to certain *Lycopersicon* accessions than other isolates (Lebeda and Mieslerová 2000), and that the Japanese isolate KTP-01 is virulent to a resistant cultivar bred in the Netherlands (Kashimoto et al. 2003).

Resistance to *O. neolycopersici*

Apart from a few modern resistant cultivars, most of the tomato cultivars are susceptible to *O. neolycopersici.* Promising sources of resistance to *O. neolycopersici* are available in different *Lycopersicon* and *Solanum* species (Lindhout 1994a&b, Mieslerová et al. 2000). The resistance in *L. hirsutum* G1.1560 and G1.1290 is controlled by two dominant genes, *Ol-1* and *Ol-3*, which map on the long arm of Chromosome 6 (Huang et al. 2000c, Van der Beek et al. 1994). The resistance in *L. esculentum* var *cerasiforme* LA1230 is controlled by a recessive resistance gene *ol-2* that maps on Chromosome 4 (Ciccarese et al. 1998, De Giovanni et al. 2004). The resistances in several other accessions may be different and were still unknown at the start of this PhD project.

Plant resistance responses to powdery mildew (*Erysiphaceae)* infection are mainly associated with a hypersensitive necrotic response (HR) and papillae formation. HR is defined as local plant cell death in response to pathogen attack, and papillae are cell wall appositions that are deposited on sites of attempted fungal appressorial penetration (Israel 1980, Heath 1981). Histological studies showed that the resistance to *O. neolycopersici* in wild tomato species is mainly associated with HR, although HR was not always effective to fully stop the fungal development. Papillae formation that is usually associated with prehaustorial resistance also occurred, but was not a prominent defense mechanism in wild *Lycopersicon* species (Huang et al. 1998, Lebeda et al. 2000).

Scope of this thesis

The aim of this research was to study the host-pathogen interaction of tomato (*Lycopersicon*) and tomato powdery mildew (*O. neolycopersici*). The first step was to characterize the genetic basis of the resistance of some *Lycopersicon* species and map the corresponding resistance genes on the tomato genome. The next step was to develop a set of near isogenic lines (NILs) with different qualitative resistance genes (*Ol*-genes) or quantitative resistance genes (*Ol*-qtls)

in a genetic background of the susceptible *L. esculentum* cv. Moneymaker. The final step was to characterize the NILs in their interaction with different isolates of *O. neolycopersici* and to study the resistance mechanisms associated with the different *Ol*-genes.

In **Chapter 2,** the *O. neolycopersici* resistance originating from *L. parviflorum* G1.1601 was characterized. Map positions of three QTLs (*Ol*-qtls) involved in the quantitative resistance from *L. parviflorum* were presented, and evidence was provided for co-localization of two *Ol*-qtls with two qualitative resistance genes involved in tomato powdery mildew resistance.

In Chapter 3, a set of simple PCR markers was generated (http://www.dpw.wau.nl/pv/CAPStomato/), which were derived from tomato RFLP probes (Tanksley et al. 1992) and are useful for mapping studies.

In **Chapter 4**, a study of the resistance to *O. neolycopersici* in *L. peruvianum* LA2172 was presented. *L. peruvianum* is an out-crossing species that is reproductively isolated from the "*esculentum* complex" by severe crossing barriers. Complexities like multi-allelism and distorted segregation hampered mapping of the *O. neolycopersici* resistance in *L. peruvianum* LA2172. Consequently, multi-allelic single locus markers were generated and the pitfalls of the exclusive use of bi-allelic markers were illustrated.

Chapter 5 focuses on the genomic distribution of five dominant *Ol*-genes. The experimental approach of using a set of common locus-specific PCR markers in different mapping populations segregating for *Ol*-genes was used, which allowed us to generate an integrated map consisting of DNA markers and different *Ol*-genes. Remarkably, all the dominant *Ol*-genes mapped so far are located on tomato Chromosome 6 and are organized in three genetic loci.

In **Chapter 6**, the generation of NILs that contain different *Ol*-genes (*Ol-1, ol-2, Ol-3, Ol-4, Ol-5* or *Ol-6*) was described. The race specificity of the resistance conferred by the *Ol*-genes was determined by testing the NILs with local isolates of *O. neolycopersici* in different geographic places. Moreover, the resistance mechanisms associated with the *Ol*-genes were studied microscopically.

Chapter 7 is a general discussion of this thesis. Several aspects are discussed, such as the organization and evolution of the *Ol*-genes, hot spots of resistance, race-specific resistance and relations with different resistance mechanisms, as well as the implications of the results for tomato breeding.

Chapter 2

QTLs for tomato powdery mildew (*Oidium neolycopersici*) resistance in *Lycopersicon parviflorum* G1.1601 co-localize with two qualitative powdery mildew resistance genes

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ABSTRACT

Tomato (*Lycopersicon esculentum*) is susceptible to the powdery mildew, *Oidium neolycopersici*, but several wild relatives like *L. parviflorum* G1.1601 are completely resistant. An F_2 population from a cross of *L. esculentum* cv. Moneymaker x *L. parviflorum* G1.1601 was used to map the *O. neolycopersici* resistance by using AFLP markers. The resistance was controlled by three quantitative trait loci (QTLs). *Ol-qtl1* is on Chromosome 6 in the same region as the *Ol-1* locus, which is involved in a hypersensitive resistance response to *O. neolycopersici*. *Ol-qtl2* and *Ol-qtl3* are located on Chromosome 12, separated by 25 cM, in the vicinity of the *Lv* locus conferring resistance to another powdery mildew species, *Leveillula taurica*. The three QTLs, jointly explaining 68% of the phenotypic variation, were confirmed by testing F_3 progenies. A set of PCR based CAPS/SCAR markers was generated for efficient monitoring of the target QTL genomic regions in marker assisted selection. The possible relationship between genes underlying major and partial resistance for tomato powdery mildew is discussed.

INTRODUCTION

Tomato powdery mildew (*O. neolycopersici*) has become a globally important fungus since 1986, when it was reported in the Netherlands (Paternotte 1988), and later on quickly spread over all tomato growing areas in the world. Most modern tomato cultivars are susceptible, but resistance has been found in many *Lycopersicon* species (Ciccarese et al. 1998, Lindhout et al. 1994a). Resistance in *L. hirsutum* G1.1560 and G1.1290 is controlled by incompletely dominant genes, *Ol-1* and *Ol-3*, respectively, which map on the long arm of Chromosome 6 and are probably allelic (Huang et al. 2000c, Van der Beek et al. 1994). In the evaluated wild accessions of *L. peruvianum* and *L. hirsutum*, resistance is mainly associated with a hypersensitive response (HR), while in *L. parviflorum* G1.1601, the association of resistance with HR was not as strong as in other accessions (Huang et al. 1998). This suggests that resistance in G1.1601 may be partly due to a different mechanism than HR. In addition, earlier studies showed that the inheritance of resistance in G1.1601 is polygenic or recessive (Lindhout et al. 1994b).

Another species of tomato powdery mildew, *Leveillula taurica*, has been reported to occur in subtropical regions. The mycelium of *L. taurica* grows into the leaf and is visible on the lower side of the leaf. It is different from *O. neolycopersici* that grows mainly on the upper epidermis and usually does not penetrate the leaf (Lindhout et al. 1994a). A single dominant gene, *Lv*, was identified in *L. chilense* and introduced into the cultivated tomato. The *Lv* locus is mapped on Chromosome 12 flanked by RFLP markers CT121 and CT129, and is currently the sole source of resistance to *L. taurica* (Chunwongse et al. 1997).

Monogenic resistance is often race-specific and associated with HR, a commonly occurring defense mechanism in plants. During the last 10 years, many monogenic resistance genes (R genes) have been identified and dozens have been isolated (Dangl and Jones 2001). Protein structural similarities of the cloned R genes have allowed isolation of structurally related sequences referred to as resistance gene analogues (RGAs). Genomic clustering of R genes and RGAs is observed either at complex loci or at larger genomic regions where numerous R genes may span a few to 20 cM (as reviewed by Hulbert et al. 2001). In contrast to the rapidly increasing knowledge on monogenic resistance, little is known about the molecular basis of quantitative resistance, for which Quantitative Trait Loci (QTL) for resistance play a role in plant-pathogen interactions. Comparative studies of genetic map positions between QTLs for resistance and R genes may provide evidence for possible genomic and functional relationships between genes underlying monogenic and quantitative resistance. In many cases, the map positions of QTLs for resistance overlap with major resistance genes, RGAs or plant general defense genes (Faris et al. 1999, Geffroy et al. 2000, Grube et al. 2000, Pan et al. 2000, Pflieger et al. 2001a&b, Rouppe van der Voort et al. 1998). This may occur by chance, due to clustering of genes or may be a consequence of pleiotropic effects. The latter may indicate a

potential similar molecular basis of quantitative and qualitative resistance genes. By studying the organization of resistance genes in the potato genome, Gebhardt and Valkonen (2001) proposed that the molecular basis of quantitative resistance in potato is based on genes having structural similarity with cloned R genes and on genes involved in the defense response. However, there are also examples of QTLs that do not overlap with the positions of known R genes, RGAs or plant general defense genes (Geffroy et al. 2000, Qi et al. 1998, Van Berloo and Lindhout 2001). This may indicate that QTLs for resistance harbor unique resistance gene families or their regulatory loci.

The aim of our research is to investigate the genetic basis and the molecular mechanism of *O. neolycopersici* resistance from *L. parviflorum* G1.1601. In this paper, map positions of three QTLs involved in the quantitative resistance from *L. parviflorum* are presented, and evidence is provided for co-localization of two QTLs with R genes involved in tomato powdery mildew resistance.

MATERIALS AND METHODS

Plant and fungal materials

An F_2 population of 209 plants derived from an interspecific cross between individual plants of the susceptible *L. esculentum* cv. Moneymaker and the resistant accession *L. parviflorum* G1.1601 was used to study the inheritance of the resistance. All F_2 plants were selfed, but only 171 F_2 plants resulted into F_3 progenies with sufficient numbers of seeds.

The pathogenic fungus *O. neolycopersici*, which originated from infected commercial tomato plants (Lindhout et al. 1994a), was maintained on MM plants in a greenhouse compartment at 20±3 °C with 70±15% relative humidity (RH).

Disease test

A disease test was performed by spraying one-month-old tomato plants with a suspension of $2x10^4$ conidia.ml^{-1}. The inoculum was prepared by washing conidial spores from freshly sporulating leaves of heavily infected MM plants in tap water and was used immediately. For the disease test of the F_2 population, experiments were carried out according to a randomized block design. Six blocks were used, each containing two plants of each parent and of the F_1, and 34-35 F_2 plants. For the F_3 lines testing, a complete randomized block design was used. Each of two blocks contained the 10 F_3 lines (24 plants per line) and two parents (24 plants each). The inoculated plants were grown in a greenhouse at 20±3°C with 30-70% RH.

The fungal growth was evaluated at 11, 14 and 19 days post inoculation (dpi) for the F_2 population, at 14 and 18 dpi for the F_3 progenies. A disease index was used where 0 = no sporulation; 1 = slight sporulation, but less than 5% foliar area affected; 2 = moderate sporulation, 5-30% foliar area affected; 3 = abundant sporulation, more than 30% foliar area affected.

AFLP analysis

Total DNA was extracted from frozen young leaves as described by Van der Beek et al. (1992). About half (n =104) of the F_2 population was selected for AFLP analysis based on the following criteria: 1) equal representatives of the three disease classes ($0 \leq DI \leq 1$, $1< DI \leq 2$, $2< DI \leq 3$), 2) large amount of DNA extracted per F_2 plant, 3) large number of F_3 seeds obtained.

The AFLP procedure was performed as described by Vos et al. (1995) with some modifications according to Qi et al. (1997). Restriction enzymes, adapters and primers used are listed in Table 1. The following primer combinations were used: P11M48, P14M49, P14M50, P14M60, P14M61, P14M62, P15M48, P18M50, P18M51, P22M50, P22M60, E35M48 and E39M50. The underlined primer combinations have also been used for the tomato genetic map by Haanstra et al. (1999b). The 5'end of the selective *Eco*–primer was labeled with radioactivity ^{33}P and the selective *Pst*-primer labeled with IRD700 or IRD800. Electrophoresis and gel analysis for ^{33}P-labeled AFLPs was done as described by Vos et al. (1995), and IRD-labeled AFLPs was analyzed on a LI-COR 4200 DNA sequencer, essentially following the method published by Myburg and Remington (2000).

SCAR and CAPS analysis

CAPS (cleaved amplified polymorphic sequence) marker *Tm2a* was used as described by Sorbir et al. (2000), and primers for CAPS *Aps1* and CP60 have been published by Van Daelen (1995) and Bendahmane et al. (1997), respectively. Other PCR-based CAPS and SCAR (sequence characterized amplified region) markers were generated from RFLP markers previously mapped by Tanksley et al. (1992). The sequences of the RFLP markers were either available as expressed sequence tags (ESTs) in the SolGenes database or obtained by sequencing (Baseclear, Leiden, the Netherlands) bacterial clones containing the RFLP probes obtained from Cornell University, Ithaca, New York. Primers (Table 2) were designed by using the DNAstar software package and used to amplify the genomic DNA of the two parents (MM and G1.1601). If no polymorphism between the two parents was observed, the amplification products were subjected to restriction analysis with different restriction enzymes or were sequenced (Baseclear, Leiden, the Netherlands) to detect a polymorphism. Polymorphisms detected by sequencing, for which no diagnostic enzymes were available, were, if possible, converted into

Table 1. Sequences of AFLP primers and adapters

Primers/adapters		**Sequences**[a]
*Mse*I adapter		5'-GACGATGAGTCCTGAG-3'
		3'-TACTCAGGACTC AT-5'
M00 (universal primer)		GATGAGTCCTGAG TAA
*Mse*I + 1 primer M02		M00 + C
*Mse*I + 3 primers	M48	M00 + CAC
	M49	M00 + CAG
	M50	M00 + CAT
	M51	M00 + CCA
	M60	M00 + CTC
	M61	M00 + CTG
	M62	M00 + CTT
*Eco*I adapter		5'-CTCGTAGACTGCGTACC-3'
		3'-CTGACGCATGG TTAA-5'
E00 (universal primer)		GACTGCGTACC AATTC
*Eco*RI + 1 primer E01		E00 + A
*Eco*RI + 3 primer	E35	E00 + ACA
	E39	E00 + AGA
*Pst*I adapter		5'-CTCGTAGACTGCGTACATGCA-3'
		3'-CATCTAGACGCATGT-5'
P00 (universal primer)		GACTGCGTACATGCAG
*Pst*I + 1primer P01		P00 + A
*Pst*I + 2 primer	P11	P00 + AA
	P14	P00 + AT
	P15	P00 + CA
	P18	P00 + CT
	P22	P00 + GT

[a] DNA sequences are always from 5' to 3' orientation unless indicated otherwise.

dCAPS (derived CAPS) markers according to the method described by Neff et al. (1998). Each PCR reaction (25 µl) contained 100 ng of genomic DNA, 1x PCR-reaction buffer, 50 ng of each forward and reverse primer, 0.2 mM dNTPs and 0.5 unit Taq-polymerase in demi water. PCR conditions were: 1 cycle at 94°C for 5 min, followed by 35 cycles of 30 s at 94°C, 30 s at annealing temperature (see Table 2), 45 s at 72 °C and a final extension of 7 min at 72 °C. The PCR products were separated on 1.5% agarose gel stained with Ethidium Bromide and DNA fragments were visualized by UV light. About 3 µl of crude PCR product was digested in a total volume of 15 µl for 2-3h with 1 unit of the appropriate restriction endonuclease. Buffers and

temperature were as described by the manufacturer. After digestion, DNA fragments were separated on 2-3% agarose gel and visualized by UV light.

For the conversion of AFLP to CAPS/SCAR markers, ^{33}P-labeled amplification products were excised from a dried AFLP gel and re-suspended in 50 µl H_2O. The AFLP fragments were re-amplified using the corresponding unlabeled AFLP primers based on standard conditions as described above with an annealing temperature of 56°C. The PCR products were cloned using PGEM-T Easy vectors and transformed into DH5α^{TM} Competent Cells. To ascertain that the proper AFLP fragment was isolated, DNA samples of four colonies for each AFLP marker were sequenced (Greenomics, Wageningen, the Netherlands). New primers internal to the AFLP selective primers were designed to amplify the genomic DNA of the two parents (MM and G1.1601). The primer design, PCR and restriction analysis were carried out as described previously for the conversion of ESTs.

Map construction and QTL mapping

JOINMAP 2.0 (Stam and Van Ooijen, 1995) was used to generate a genetic map applying the Kosambi's mapping function. QTL mapping was performed using MapQTL 4.0 (Van Ooijen and Maliepaard 1996). A logarithm of odds (LOD) threshold value of 3 was set for declaring a QTL in Interval Mapping (IM; Van Ooijen, 1999). After IM, a two-LOD support interval was taken as a confidence interval for a putative QTL (Van Ooijen 1992). Markers at the LOD peaks were taken as co-factors for running the multiple QTL mapping program (MQM) to verify the results of IM.

RESULTS

Inheritance of resistance to O. neolycopersici from L. parviflorum G1.1601

A disease test was performed on the F_2 population (n = 209) of *L. esculentum* cv. Moneymaker (here after referred to as MM) x *L. parviflorum* G1.1601 (here after referred to as G1.1601) to assess the inheritance of resistance to *O. neolycopersici*. All plants were evaluated for the degree of sporulation expressed as disease index (DI) at a scale from 0 to 3. Plants of the resistant parent G1.1601 were either immune (DI = 0) or were slightly infected (scored as 1), while all plants of the susceptible parent MM were heavily infected (scored as 3, Fig. 1). The F_1 showed predominantly an intermediate DI of 1 or 2 and the F_2 plants were normally distributed over the DI classes 0 to 3 with a mean DI value of 1.8 (Fig. 1). Thus no monogenic model for the inheritance of resistance could be deduced. This result indicates that the resistance to

O. neolycopersici in G1.1601 is quantitatively inherited and is likely to be controlled by more than one gene.

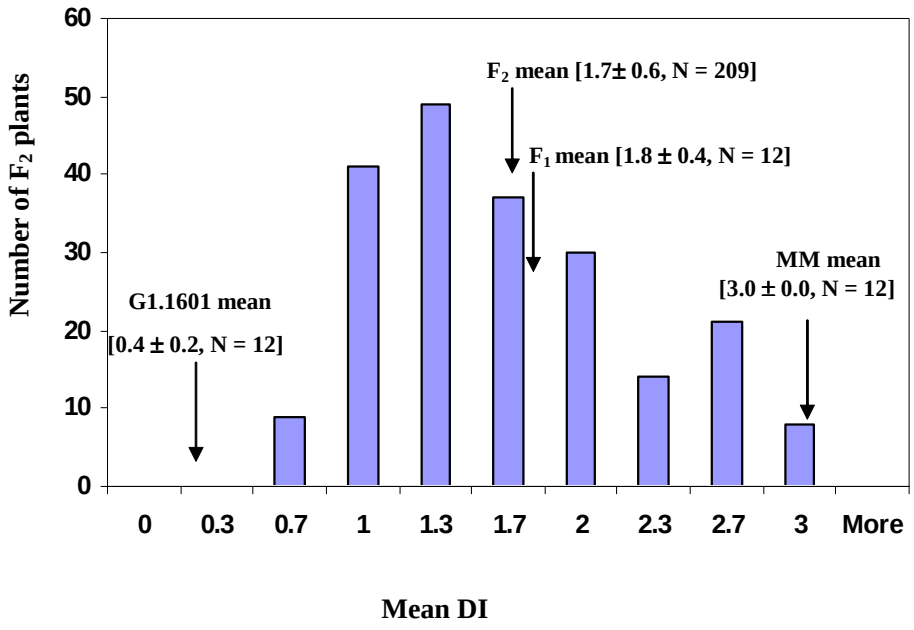

Fig. 1. Frequency distribution of the mean value of disease index (DI) for resistance to *O. neolycopersici* in an F_2 population derived from the cross *L. esculentum* cv. Moneymaker (MM) x *L. parviflorum* G1.1601. The mean DI was an average of DI evaluated at 11, 14 and 19 days post inoculation. The mean DI values of the two parents, F_1, and the overall F_2 population are indicated by arrows. The population sizes are indicated between brackets.

Molecular markers and map construction

AFLP analysis was performed to obtain a sufficiently large set of markers to generate a genetic linkage map from the F_2 population of MM x G1.1601 (n = 104, see Material and Methods). By using 14 *Pst*I/*Mse*I and two *Eco*RI/*Mse*I primer combinations, in total 318 markers were obtained; 154 were MM specific and 164 were G1.1601 specific. Initially, all markers were scored dominantly, but 34 markers could be at least partially scored co-dominantly using the Quantar-Pro™ software (Keygene, Wageningen, the Netherlands). To improve the linkage map, 25 PCR based markers were added. These markers were mainly co-dominant and with known map positions on Chromosome 2, 4, 6, 9, 11 and 12 of a *L. esculentum* x *L. pennellii* map (Tanksley et al. 1992, markers linked to the QTLs are listed in Table 2). The co-dominant

Table 2. Primer sequences and PCR conditions for the CAPS/SCAR markers

Name[a]	Chromo-some location	Marker type	Primer sequence (5'-3')	Tm[b] (°C)	PCR product size (bp)	Restric-tion enzyme	Original source	
							marker type	marker name[a]
Aps1	6	CAPS	atggtgggtccaggttataag cagaatgagcttctgccaatc	56	1000	*Sau*96 I	CAPS	*Aps1*[c]
dct21	6	dCAPS	ctttggtggttcagctattaatcc catttccaacaaaccattcttccc	50	185	*Msp* I	RFLP	CT21
tg25	6	CAPS	caacagctgccacaaacact agtttggtgcttcatgcaaa	56	500	*Hae* III	RFLP	TG25
dct136	6	dCAPS	cgaagtgtcggatccgaaggcttt aacacaatcggaaaaaa	47	180	*Xmn* I	RFLP	CT136
ct129	12	CAPS	tctgtgatctgatatgtctaag ctcctggggtaagtttc	50	1500	*Hinf* I	RFLP	CT129
ct99	12	CAPS	atctaaaaacacgccaataatct ggaccatcggagggagcac	54	309	*Rsa* I	RFLP	CT99
Y258	12	CAPS	gtaattccaaaaagtgaggt ttgcgtctagagttatttt	50	136	*Mbo* I	AFLP	P11M48-285
B432U	12	dominant SCAR	tcagaaagggaagaatcaag cccgatccaatgttatgtctgaa	56	300	-	AFLP	E39M50-432
tg111	12	co-dominant SCAR	tgccaacccggacaaaga tggggaagtgattagacaggaca	54	399+1000	-	RFLP	TG111

[a] The RFLP-marker names are written in capital (like TG25), and the corresponding CAPS/SCAR Marker name in small case (like tg25).

[b] PCR annealing temperature.

[c] Primers for *Aps1* was published by Van Daelen (see SCAR and CAPS analysis in Materials and Methods

AFLP and PCR based markers served as bridges in map construction to merge the dominant markers into an integrated map comprising markers of both parents. Fifteen linkage groups were identified, covering a total genetic length of 761 cM. It has been reported that co-migrating AFLP bands within a species are generally allele specific (Qi et al. 1998, Haanstra et al. 1999b, Rouppe van der Voort et al. 1997). Therefore, 32 MM specific AFLP markers that were in common with the markers in the genetic map published by Haanstra et al. (1999b), and the locus specific SCAR and CAPS markers served as anchor markers to assign linkage groups

to chromosomes. Consequently, 10 of the 12 chromosomes could be identified, but not Chromosomes 5 and 10 for which anchor markers were lacking. Clustering of markers rarely occurred in this map since the majority of the AFLP markers were *Pst*I/*Mse*I markers that cluster less than *Eco*RI/*Mse*I markers (Qi et al. 1998, Haanstra et al. 1999b). Compared with the maps published by Tanksley et al. (1992) and Haanstra et al. (1999b), the relative order of the anchor markers was consistent, and six out of the 12 tomato chromosomes (Chromosome 1, 2, 4, 6, 11 and 12) were well saturated with markers (data not shown).

QTL mapping

By applying Interval Mapping (IM), three QTLs for resistance to *O. neolycopersici* were identified, and all resistance alleles originated from the resistant parent G1. 1601. All the three QTLs showed up at all infection stages (11, 14 and 19 days post inoculation) with similar effects, thus, there was no evidence for time-dependent QTLs. One QTL designated as *Ol-qtl1* mapped on Chromosome 6 with a highest LOD value at the CAPS marker tg25, coinciding with a genomic region containing *Ol-1/Ol-3* from *L. hirsutum* (Fig. 2, Table 3, also see Huang et al. 2000c for *Ol-1/Ol-3* map position). The other two LOD peaks were on Chromosome 12, at a distance of 25 cM from each other (Fig. 2, Table 3). To verify whether these two peaks corresponded to two linked QTLs, cofactors at the two peak positions were chosen for MQM mapping. Again, two clearly distinct LOD peak profiles were obtained with a similar LOD value above 3 (data not shown). Thus, MQM confirmed the presence of two linked QTLs, designated as *Ol-qtl2* and *Ol-qtl3*, on Chromosome 12. *Ol-qtl2* was flanked by CAPS markers ct99 and ct129. Remarkably, the RFLP marker CT129 is also closely linked to the *Lv* locus, a major tomato resistance gene to another powdery mildew species, *Leveillula taurica* (Chunwongse et al. 1997).

Table 3. The three QTLs associated with resistance to *O. neolycopersici*, detected by Interval Mapping in an F_2 population of *L. esculentum* cv. Moneymaker x *L. parviflorum* G1.1601

QTL name	Chromosome	Nearest marker	LOD peak value	Variation explained (%)	Additive effect
Ol-qtl1	6	tg25	3.8	16.1	0.34
Ol-qtl2	12	P18M51-701c	7.1	29.5	0.42
Ol-qtl3	12	B432u	5.0	22.3	0.45

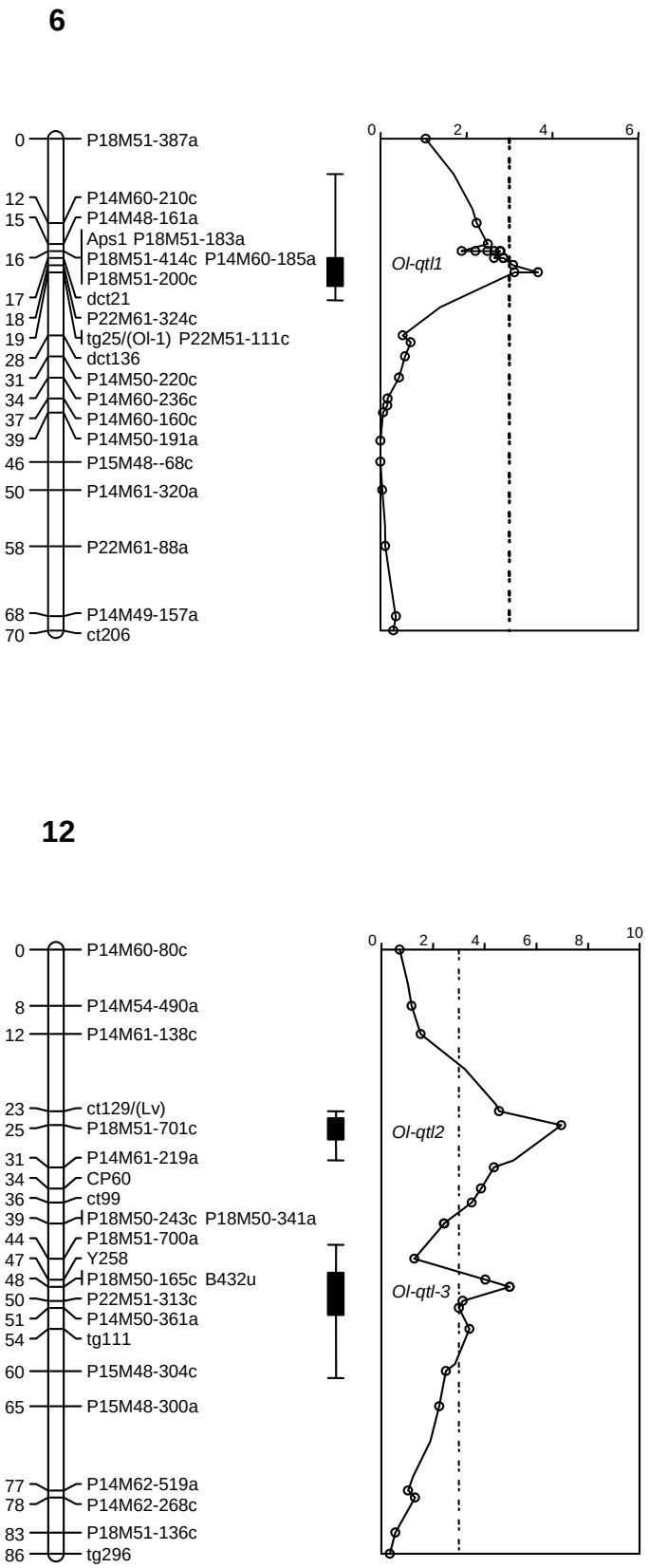

Fig. 2. The positions of QTLs for *O. neolycopersici* resistance are shown with the linkage maps of Chromosome 6 and 12, which are derived from the F_2 population of the *L. esculentum* cv. Moneymaker x *L. parviflorum* G1. 1601. Map positions are given in cM. AFLP marker loci are assigned with primer-combination name and fragment size followed by a or c, indicating a MM or G1.1601 specific marker, respectively. Bars indicate the QTL intervals for which the inner bar shows a one-LOD support interval and the outer bar shows a two-LOD support interval. Graphs show the QTL likelihood profiles for interval mapping. The LOD threshold value of 3.0 is shown as a dotted line.

Effects of the identified QTLs on the level of resistance

By using co-dominant markers, both dominance and additive effects could be detected in this study. All three QTLs showed only additive effects (0.34, 0.42 and 0.45, respectively) and jointly explained 68% of the total phenotypic variation (Table 3). Assuming absence of epistasis and dominance, with an almost equal additive effect of each QTL resistance allele on resistance, a linear relationship between *O. neolycopersici* resistance and the number of resistance allele(s) at QTLs was expected. To test this hypothesis, the 104 F_2 plants were grouped according to the presence of the number of putative QTL resistance alleles in these plants. A two-LOD support interval was taken as a confidence interval for the position of each QTL (van Ooijen et al. 1992), and markers flanking and within this region were taken as indicators for the presence or absence of the corresponding QTL resistance allele(s). We preferably used the co-dominant PCR based markers (generated from RFLP markers) that were closely linked to the QTLs (Table 2). In addition, two AFLP markers linked to the QTLs were converted into CAPS or SCAR (Table 2). Seventy-three out of the 104 F_2 plants could clearly be genotyped without any recombination in the QTL intervals and were grouped according to the number of QTL resistance alleles. Fitting a quadratic model revealed that the quadratic term was not significant ($P = 0.31$), which indicated absence of epistatic interaction between the QTLs. An obvious linear correlation ($R^2 = 0.95$) was observed between increasing numbers of QTL resistance alleles and decreasing DI values (Fig. 3). A similar linear relationship ($R^2 = 0.89$) was observed in BC_1 lines containing one to three of the QTL resistance allele(s) derived from different F_3 plants (data not shown). If only estimated additive effects of the three QTL resistance alleles are taken into account, the predicted DI difference between the two parents would be 2.42 (Table 3), which is close to the observed DI difference between the two parents of 2.66 (Fig. 1). This implies that the detected three QTLs accounted for nearly the complete phenotypic difference between the two parents, suggesting that most of the genetic variation is explained by these QTLs. However, hardly any F_2 plant with all the QTL resistance alleles was as resistant as the resistant parent G1.1601 (Fig. 1 and Fig. 3), indicating some other minor QTLs might have escaped detection or morphological variations among the F_2 plants might complicate the evaluation of the resistance. To verify the effects of the QTLs, a disease test was performed on F_3 progenies. In total, ten F_3 lines were selected that had QTL genotypes like the two parents (MM and G1.1601) or the F_1 (Table 4). As expected, segregation of resistance (DI from 0 to 3) was observed mainly in the F_3 progenies from the F_2 plants with a heterozygous QTL genotype. The average DI for this group was 1.2, similar to the predicted additive effects of the QTLs. The F_3 progenies from the F_2 plants carrying six QTL resistance alleles had a mean DI of 0.5, which is similar to the DI of 0.6 for the resistant parent G1.1601 in the same experiment. The F_3 progeny from one F_2 plant, which was devoid of any QTL resistance allele, showed a slightly

lower DI than MM. The difference between the average DI of F_3 lines containing zero and six QTL resistance alleles was 2.0, which again is close to the DI difference (2.4) between the parents as controls (Table 4). This is in agreement with the results from the F_2 population. In conclusion, the three QTLs jointly explained most of the resistance in the resistant parent *L. parviflorum* G1.1601.

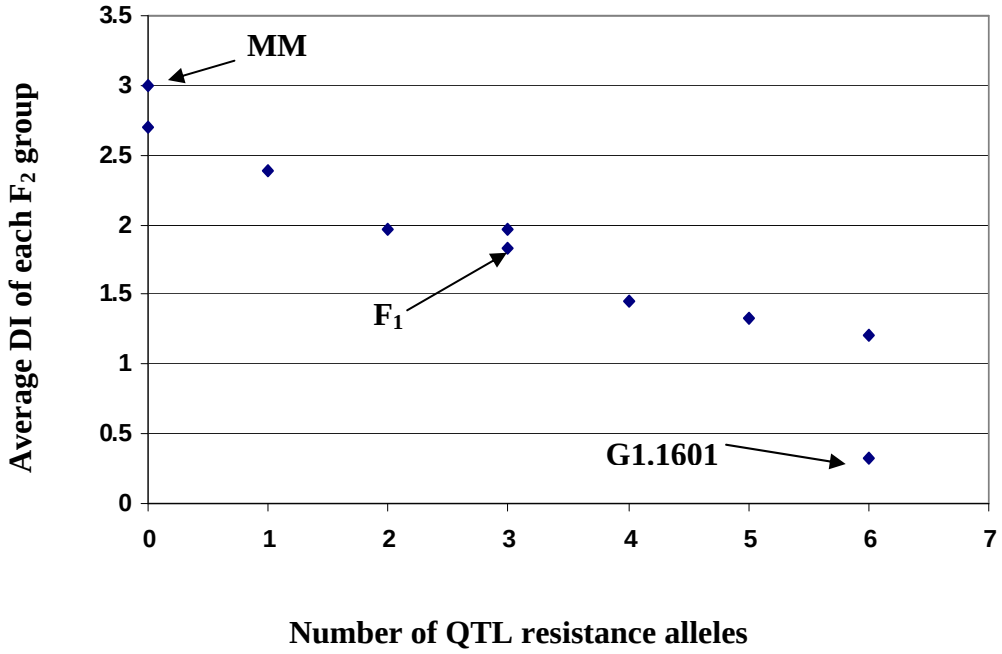

Fig. 3. Average DI plotted against the number of QTL resistance alleles present in the F_2 plants of each group. The mean DI was an average of DI evaluated at 11, 14 and 19 days post inoculation (dpi). The mean DI values of the two parents, F_1 and the overall F_2 population mean are indicated by arrows.

Table 4. The DI value of the tested F_3 lines. The DI average was a mean of DI evaluated at 14 and 18 days post inoculation

Plant	No. of QTL resistance alleles in the F_2 and the parents	No. of F_3 lines tested	No. of plants tested	Average DI	No. of plants in three DI classes		
					0 ≤ DI ≤ 1	1< DI ≤ 2	2< DI ≤ 3
F_3	6	4	96	0.5	80	12	4
F_3	3 (as F_1 genotype)	5	119	1.2	56	32	31
F_3	0	1	24	2.5	0	5	13
MM	0		24	3	0	0	24
G1.1601	6		24	0.6	20	4	0

DISCUSSION

The disease test on the F_2 population demonstrated that resistance to *O. neolycopersici* in G1.1601 is inherited quantitatively, unlike the dominant monogenic resistance in *L. hirsutum* G1.1290 and G1.1560 (Huang et al., 2000c, Van der Beek et al., 1994). Three QTLs were identified that jointly explained most of the total phenotypic variation with only additive effects. In the present study, evidence has been provided that *Ol-qtl2* and *Ol-qtl3* are both located on Chromosome 12 at a distance of 25 cM. To verify this, next progenies are generated to dissect these two QTLs by selecting recombinants between linked molecular markers. Giving the limitations of QTL mapping (van Ooijen 1992), it is hard to assume that not any QTL against *O. neolycopersici* has escaped our attention. However, the results of our study on the F_2 and the F_3 progenies clearly indicated that the three QTLs identified so far explain most of the resistance in *L. parviflorum*. Since quantitative resistance is generally believed to be more durable (Johnson 1981, Lindhout 2002), it would be of great interest to combine and incorporate these QTL resistance alleles into modern tomato cultivars. Therefore, the QTL linked PCR based CAPS and SCAR markers that have been generated in this study are good diagnostic markers for marker-assisted breeding.

The map positions of two QTLs co-localized with the major resistance loci for tomato powdery mildews, *Ol-1/Ol-3* on Chromosome 6 and *Lv* on Chromosome 12. The genetic interval of *Ol-qtl1* coincided with *Ol-1* and *Ol-3* genes that are possibly allelic and involved in HR resistance to *O. neolycopersici* (Huang et al. 2000c). Moreover, *Ol-qtl2* coincided with the *Lv* locus, a major tomato resistance gene against *L. taurica* (Chunwongse et al. 1997). Our observation is similar to other examples of co-localization between QTLs for resistance and major resistance genes (Caranta et al. 1997, Geffroy et al. 2000, Grube et al. 2000, Marcaewski et al. 2001). The presence of both quantitative and qualitative resistance genes in the same genomic regions is not a solid proof for allelism, since the accuracy of QTL mapping does not allow pinpointing a QTL to just one gene, but rather to a chromosomal region that may contain a multitude of genes. However, more evidence has recently accumulated that resistance loci tend to exist as complex loci containing clustered multigene families. For instance, the *I-2* locus on Chromosome 11 of tomato is involved in resistance to *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*) race 2 and belongs to the NBS-LRR class of R-genes. At this complex locus originating from *L. pimpinellifolium*, multiple functional genes have been identified. *I2C-1* and *I2C-5* can confer partial resistance and *I2C-K* appears to confer complete resistance specific for the *I-2* phenotype. A similar locus conferring only intermediate resistance to *Fol* race 2 exists in the syntenic position of *I-2* in *L. pennellii* genome (Sela-Buurlage et al. 2001). Similarly, the *Ol-qtl1* locus on Chromosome 6 in the present study may correspond to another allele of the *Ol-1* locus in the *L. hirsutum* genome. This is also in agreement with the observations that major

resistance genes, once overcome by a strain of the pathogen, might conserve some residual effects. One example is that a "defeated" rice resistance gene at *Xa4* locus acts as a QTL against a virulent strain of *Xanthomonas oryzae* pv. *oryzae* (Li et al. 1999). In addition, it has been reported that modification of a monogenic resistance gene can give rise to a partial resistance gene. For example, in flax, the insertion of the transposable element *Ac* in the promoter region of the *M* rust resistance gene results in partial resistance (Anderson et al. 1997). The wild species *L. parviflorum* G1.1601 is susceptible to *L. taurica* (PL, unpublished) and thus does not contain the *Lv* gene. Still, *Ol-qtl2* might be an ortholog of the *Lv* gene, or a modified *Lv* gene conferring partial resistance to *O. neolycopersici*.

Comparative mapping studies within the Solanaceae genus showed that resistance genes (both quantitative and qualitative) occurred at syntenic positions in cross-generic clusters more frequently than expected by chance, and often clustered genes showed specificities to related and also unrelated pathogen taxa (Grube et al. 2000). The *Lv* locus belongs to one of these cross-generic clusters on Chromosome 12, which, in addition to the *Lv* gene in tomato, harbours the resistance genes *Gpa2* and *Rx* in potato, conferring resistance to the potato cyst nematode *Globodera pallida* and potato virus X (PVX), respectively. Intriguingly, the proteins encoded by the *Gpa2* and the *Rx1* genes share an overall homology of over 88% (amino-acid identity) and belong to one class of plant resistance genes, containing a leucine-zipper, nucleotide-binding site and leucine-rich repeat (LZ-NBS-LRR, Van der Vossen et al. 2000). This suggests that relatively small changes in resistance gene sequence can lead resistance against entirely different pathogen species (Wang et al. 1998, Ellis et al. 1999). Another even more extreme example is the gene *Mi* on Chromosome 6 in tomato that renders the plant resistant to a nematode and to an aphid (Rossi et al. 1998), indicating that the identical gene sequence may be involved in resistance to very different organisms. In the present study, we mapped the *Ol-qtl2* to the cross-generic cluster containing *Lv*, *Gpa2* and *Rx* loci. Recently, nucleotide-binding site (NBS) homologues have been mapped to this specific genomic region in tomato (Pan et al. 2000, Grube et al. 2000, Zhang et al. 2002). Therefore, *Ol-qtl2* might be an ortholog of the *Gpa2/Rx* gene belonging to NBS homologues. To gain more knowledge about the molecular basis underlying quantitative resistance, our research is aimed to clone these *Ol*-genes and the QTLs and to study the resistance mechanism regulated by them.

Upon microscopic observation, HR is reported to be the major mechanism of resistance against *O. neolycopersici* in *Lycopersicon*. However, the resistance in *L. parviflorum* G1.1601 was less clearly associated with HR than that in *L. hirsutum* G1.1290 and G1.1560, suggesting that a different resistance mechanism may occur (Huang et al. 1998). *Ol-qtl3* may be a good candidate for a gene that is involved in an alternative resistance mechanism different from HR, since as far as we know, it does not coincide with an *Ol*-locus. Resistance mechanisms

conferred by the QTLs and *Ol*-loci will be characterized by gene-expression studies at the molecular level and by detailed histological analysis, using near isogenic lines (NILs) which genetically differ only for presence of the QTLs or *Ol*-loci. Quantitative resistance is frequently presumed acting in a race non-specific manner; thus, these QTLs will be tested with isolates from several parts of the world to check whether they confer broad-spectrum resistance. We expect that our study will result in a model in tomato that allows understanding of the potential relationship between genes underlying complete and partial resistance and the respective molecular mechanism.

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Chapter 3

A set of simple PCR markers converted from sequence specific RFLP markers on tomato Chromosomes 9 to 12

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ABSTRACT

A set of 24 simple PCR markers was generated for tomato Chromosomes 9, 10, 11 and 12. Polymorphism was sought for between *Lycopersicon esculentum* and one of six other *Lycopersicon* species (*L. Parviflorum, L. cheesmanii, L. hirsutum, L. pennellii, L. peruvianum,* and *L. chilense*). PCR primers, which were designed from mapped RFLP probes, were used to amplify genomic DNA of the different species and the PCR amplification products were screened for polymorphism by testing restriction enzymes. With this approach, 24 (71%) of the 34 selected RFLPs were converted into simple PCR markers. By using a reference population, the map positions of these markers relative to the original RFLP markers were verified. These markers are locus specific and can be efficiently used for alignment of linkage maps, mapping target genes and marker assisted selection.

INTRODUCTION

The amplified fragment length polymorphism (AFLP) technology is a powerful method that provides a possibility to generate a virtually infinite number of markers without any prior sequence information of a genome (Vos et al. 1995). In our laboratory, AFLP markers are predominantly used in the construction of genetic linkage maps, e.g. in *Lycopersicon* species in tomato (Bai et al. 2003, Bonnema et al. 2002, Haanstra et al. 1999b). One drawback of AFLP markers is the limitation in allele comparisons among different mapping populations since AFLP markers are unable to transfer linkage information between species. Compared to the anonymous AFLP markers, RFLP markers are locus-specific and allow more efficient cross-mapping in related species for determination of synteny. However, RFLP analysis is expensive and laborious, which limits its use in practice. So, there is a strong need for markers that are robust, easy and cheap, and that allow cross-mapping in related species, such as simple PCR markers in forms as cleaved amplified polymorphic sequence (CAPS) markers and sequence characterized amplified region (SCAR) markers.

In tomato, more than 1000 RFLP markers have been mapped by Tanksley et al. (1992). Sequence information for many of these RFLP probes is available. Some of these RFLP probes were recently recognized as conserved ortholog sets (or COS markers), which allows assessment of syntenic relationships between the tomato and the *Arabidopsis* genome (Fulton et al. 2002). In the present study, a set of these RFLP markers (n = 34) covering tomato Chromosomes 9, 10, 11 and 12 were converted into simple PCR markers (CAPS and SCAR). These PCR markers were applied to seven accessions from different *Lycopersicon* species that are currently studied in our laboratory, namely *L. esculentum* cv. Moneymaker, *L. parviflorum* G1.1601, *L. cheesmanii* G1.1615, *L. hirsutum* LA1777, *L. pennellii* LA716, *L. peruvianum* LA2157, and *L. chilense* G1. 1556.

RESULTS AND DISCUSSION

Selection of the RFLP probes

Thirty-three RFLP makers with known sequences (preferably above 200 bp) were selected from a *L. esculentum* x *L. pennellii* map (Tanksley et al. 1992). These RFLPs are located on tomato Chromosomes 9, 10, 11 and 12 (Fig. 1), which comprised of 10 TG, 21 CT and 2 CD probes. TG probes are derived from tomato genomic DNA clones, CT and CD probes from tomato cDNA clones. In addition, the sequence U38666 was included (hereafter mentioned as a RFLP probe from a cDNA clone, which is an expressed sequence tag (EST) on Chromosome 9 obtained from GenBank database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi).

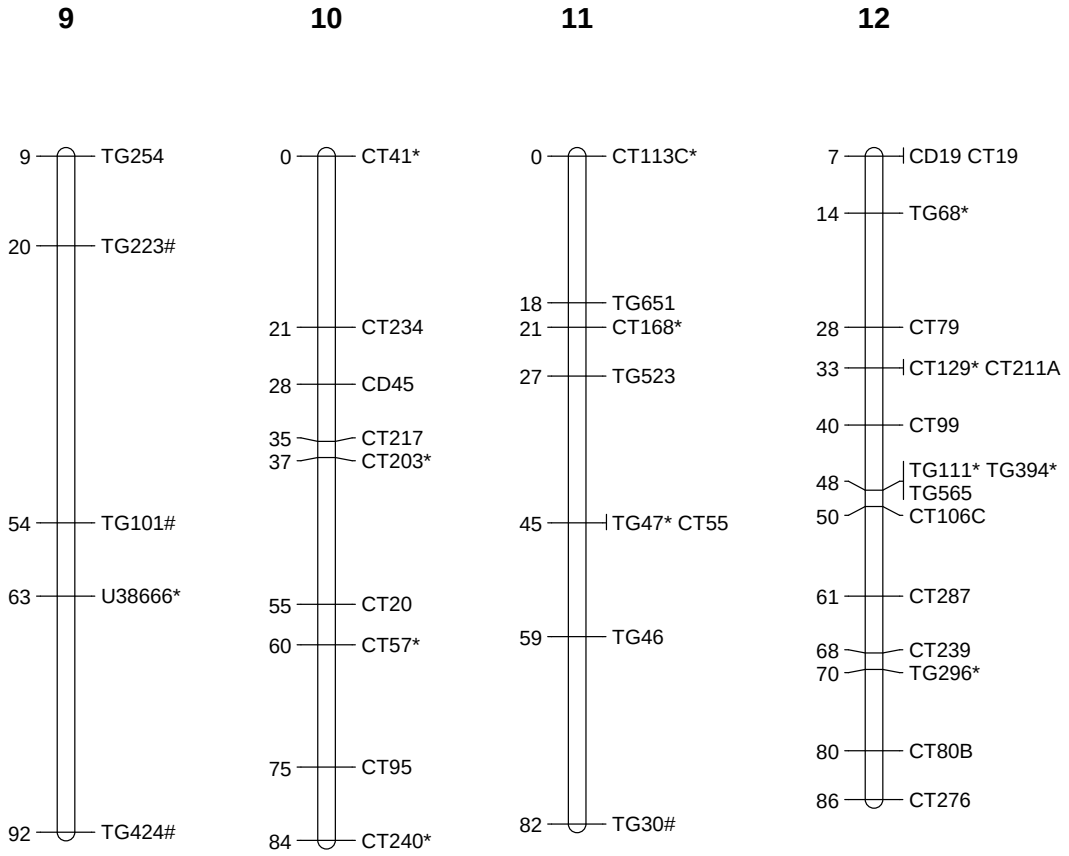

Fig. 1. Linkage map for tomato Chromosomes 9, 10, 11 and 12 showing relatively map positions of RFLP markers (coded as TG, CD and CT), which were retrieved from Tanksley et al. (1992). One EST, U38666, was obtained from GenBank and mapped on Chromosome 9. The map distances (in cM) are indicated on the left. *, Map position of the PCR markers derived from these RFLP markers were confirmed in an F_2 population of *L. esculentum* cv. Allround x *L. pennellii* LA716. #, Indication for RFLP markers that are not selected in the conversion of simple PCR markers, but are included here to show the rest of the chromosome.

Primers design

Using the sequences obtained from the Solgenes database (http://soldb.cit.cornell.edu/), locus specific PCR primers were designed as described by Bai et al. (2003). In total, 37 primer pairs were tested on genomic DNA of the seven species (Table 1). The primer sequences are listed in Table 2 and the PCR conditions were as described by Bai et al. (2003). Annealing temperature was optimized by running a gradient PCR for each primer pair on all the species. Of the 37 primer pairs tested, 29 pairs (78%, Table 1) worked well with annealing temperatures from 50°C to 60°C, with an exception for marker ct 41 (from 45°C to 50°C). Twenty-eight primer pairs successfully amplified one single DNA fragment of the same size for all species. One primer pair (tg111) revealed an identical length polymorphism (a so-called SCAR marker) between *L. esculentum* and other wild species except for *L. cheesmanii* that gave a PCR

fragment of the same size as that in *L. esculentum.* These results indicated a high level of DNA sequence conservation among *Lycopersicon* species. Primer pairs (n = 8, Table 1) that failed in PCR amplification were all derived from cDNA clones, and possibly, the failure in PCR amplification was due to large introns in the genomic DNA. Of the 19 informative primer pairs from the cDNA clones, six pairs (32%) amplified longer DNA fragments (up to 1500 bp) than predicted.

Table 1. Information of PCR primer pairs derived from the selected RFLP probes and EST sequence

RFLP probe	Number	Number of PCR primer pairs		
		designed	amplifying a PCR fragment	failed in PCR amplification
Genomic DNA clone	10	10	10	0
cDNA clone	24	27	19	8
Total	34	37	29	8

Polymorphism detection

PCR products of identical size from genomic DNA of *L. esculentum* and the six wild species were digested with different restriction enzymes. Restriction sites were predicted from the sequence information of each RFLP probe by using DNAstar^{TM} (Lasergene, Madison, WI, U. S. A). The corresponding restriction enzymes were used for restriction analysis as described by Bai et al. (2003). The set of restriction enzymes used for each primer pair was not necessarily the same. For all the informative primer pairs (n = 28), 428 restriction reactions were tested on each species, except for *L. hirsutum* where 418 restrictions were done. Polymorphism rate (percentage of tested restriction enzymes that revealed polymorphism) of the six wild species was lowest for *L. cheesmanii,* suggesting this species is most closely related to *L. esculentum* (Table 2), which is in agreement with other studies (Alvarez et al. 2001, Marshall et al. 2001). In total, 23 (82%) of the 28 primer pairs gave CAPS markers in at least one wild species (Table 2). When compared with *L. esculentum*, 13 markers were identified in *L. parviflorum*, 18 in *L. pennellii*, 16 in *L. peruvianum*, 18 in *L. hirsutum*, 14 in *L. chilense* and only one in *L. cheesmanii*. Frequently, two PCR marker alleles were observed in *L. parviflorum* and *L. chilense* even when the CAPS marker was converted from a single copy RFLP probe, showing that the loci were probably heterozygous.

Table 2. Primer sequences, lengths of PCR products and enzymes revealing a polymorphism for CAPS/SCAR markers

Marker name[a]	Chromo-some	Primer sequence (5'-3')	Predicted PCR product length (bp)	Observed PCR product length (bp)	Anneal-ing Tm (°C)	Total No. of enzymes tested	Restriction enzymes detecting polymorphism between *L. esculentum*[c] and					
							L. cheesmanii	*L. parviflorum*	*L. pennellii*	*L. hirsutum*	*L. peruvianum*	*L. chilense*
tg254	9	ttgggaatatagtgtaggaag ctggaaaggggaaagac	374	374	55	17	-[d]	-	-	*Fok* I	*Sau*3a I	-
U38666[b]	9	agctgccgtgtcctgtatca actcatgttcacgccactttctta	593	593	55	6	-	-	*Hin*f I, *Dra* I	*Dra* I	*Dra* I	*Dra* I
ct41[b]	10	caaaaatgcaatgaaaaa ttgcggaatacactgagaa	453	453	46	17	-	-	*Hin*f I	*Hin*f I	-	*Hin*f I
cd45	10	tcacaaacaacgaacaatacaaca atccgccatagcttcatcctc	217	217	55	19	-	-	-	-	-	-
ct217	10	gatacaaagatggcacaaaacagc gacgagaacggggatggatt	301	301	56	18	-	-	-	*Bcc I*	-	-
ct203[b]	10	tagaatatgggaagcgaaatg gagaggaagcgtaatagg	311	311	56	13	-	*Mbo* II	*Mbo* II, *Rsa* I	*Hae* III, *Mbo*II, *Rsa* I	*Hae* III, *Mbo*II, *Rsa* I	*Mbo* II, *Rsa* I
ct20	10	atcccccaagctaaacatcaaact gaagccggagccgaggagacag	345	345	56	19	-	-	-	-	-	-
ct240[b]	10	atcccaagtaccctcgcattagt agccttctttgtcccatcag	239	400	56	17	-	*Hae* III, *Alu* I	*Hae* III, *Dra* I	*Hae* III, *Alu* I, *Mbo* I, *Mbo* II	*Hae* III, *Alu* I	*Hae* III, *Hinf* I *Mbo* II
ct57[b]	10	gctgcagcaagaaatacaag tccagccgtgagaaaacc	352	1000	60	10	-	*Mbo* I	*Mbo* I	Not tested	*Mbo* I	*Mbo* I, *Dra* I
ct113[b]	11	acaacgggcaacagacgcaacc agctcgaggatggccgcacttt	352	352	56	20	-	*Mbo* I, *Mbo* II	*Mbo* I, *Mbo* II, *Acc* I	*Mbo* I, *Mbo* II, *Acc* I	*Mbo* I, *Mbo* II	*Mbo* I, *Acc* I
ct168[b]	11	ggaaaaactggctgctgactactt caatcatatcgcgtacctcactaa	330	1000	60	15	-	-	*Taq* I, *Hha* I	*Taq* I	-	-
tg651	11	tgcaaaaatggcggaaaat taccccaaacataaataatc	222	222	56	10	-	-	-	-	-	-
tg523	11	atttcctggattcgttttct ctactacttcacttcctggtcat	342	342	56	25	*Mnl* I	-	*Mnl* I	*Mnl* I, *Mse* I	*Mbo* II	*Mnl* I
tg47[b]	11	cctcattgggcggtcatt tcccactccaagctatactaacaa	415	415	56	11	-	*Bgi* II	*Bgi* II, *Mse* I	*Mse* I	*Bgi* II	-

Table 2. continued

Marker name[a]	Chromo-some	Primer sequence (5'-3')	Predicted PCR product length (bp)	Observed PCR product length (bp)	Anneal-ing Tm (°C)	Total No. of enzymes tested	Restriction enzymes detecting polymorphism between *L. esculentum*[c] and					
							L. cheesmanii	*L. parviflorum*	*L. pennellii*	*L. hirsutum*	*L. peruvianum*	*L. chilense*
ct55	11	catctggtgaggcggtgaagta tccgcccaaacaaaacagtaata	402	402	56	18	-	*Alu* I	*Alu* I, *Dde* I	*Alu* I, *Dde* I	*Dde* I	*Dde* I
tg46	11	gcatttgtcgaaccgctctc attacgtgatcccaacctctga	1371	1371	52	2	-	*Dde* I	*Alu* I	-	*Alu* I	-
ct19	12	gaatcagcaatccgtaat catgcaagcaaggtccacaac	171	250	56	25	-	-	-	*Dra* I, *Mbo* I	*Dra* I	-
tg68[b]	12	tttgattacacctgcctttacata ttttgaatccccttttaccat	427	427	56	3	-	-	*Mbo* I	-	-	*Mbo* I
ct79	12	caacaacaaattcccctctc ttgtggtgtttgctgatgat	165	165	56	17	-	-	-	-	-	-
ct129[b]	12	tctgtgatctgatatgtctaag ctcctggggtaagtttc	305	1000	56	14	-	*Hinf* I	*Dde* I	*Dde* I	*Dde* I	*Dde* I, *Hind* III
ct99	12	atctaaaaacacgccaataatct ggaccatcggagggagcac	310	310	56	5	-	*Rsa* I	-	*Hinf* I	-	*Hinf* I
tg111[b]	12	tgccaacccggacaaaga tggggaagtgattagacaggaca	397	397+1000	56	0	-	-	-	-	-	-
tg394[b]	12	agcctcatgagacctacaa tacagcacaatcttctacc	322	322	56	11	-	-	*Fok* I	*Fok* I	-	*Fok* I
tg565	12	ctgcaaaacaagaatcacact tctgcgaggtaggggtaag	374	374	56	20	-	-	-	*Mbo* I	-	-
ct106	12	caggcgctcattggacatt tggcgaggatcacacttg	288	288	56	8	-	-	-	-	-	-
ct287	12	ggaagacaaccatttatttat ctggcggtgaagaagtgtc	217	217	56	24	-	-	*Mbo* II	-	*Cla* I, *Mbo* II, *Mse* I	-
ct239	12	tggaacggagtacaaaacagaaga gaatgccatcagggaaaggtaact	328	800	56	20	-	-	*Hinf* I	*Hinf* I	*Hinf* I	*Hinf* I
tg296[b]	12	tgttctgtcggcataaagt tgctaaaacggacctacaa	373	373	56	19	-	*Mbo* I	*Acc* I	-	*Mbo* I	-

Table 2. continued

Marker name[a]	Chromo-some	Primer sequence (5'-3')	Predicted PCR product length (bp)	Observed PCR product length (bp)	Anneal-ing Tm (°C)	Total No. of enzymes tested	Restriction enzymes detecting polymorphism between *L. esculentum*[c] and					
							L. cheesmanii	*L. parviflorum*	*L. pennellii*	*L. hirsutum*	*L. peruvianum*	*L. chilense*
ct80	12	ggtaattttagaagccagaa caagatgtccgaagtgaagt	239	239	56	25	-	*Hin*f I, *Hae* III	-	-	-	-
Polymorphism rate[e]							1/428	15/428	25/428	27/418	21/428	19/428

[a], The CAPS/SCAR marker names are written in lower case (e.g., tg254), corresponding to RFLP markers with the same name but written in capitals (e.g., TG254).

[b], Map position of these CAPS/SCAR markers were verified in an F_2 population of *L. esculentum* cv. Allround x *L. pennellii* LA716 (see Fig. 1).

[c], *L. esculentum* cv. Moneymaker, *L. parviflorum* G1. 1601, *L. pennellii* LA716, *L. peruvianum* LA2157, *L. hirsutum* LA1777, *L. chilense* G1. 1556 and *L. cheesmanni* G1.1615 were used in this study.

[d], No polymorphism were detected by the restriction enzymes tested.

[e], polymorphism rate = number of enzymes that give polymorphism / total number of restriction enzymes tested.

Verification of the mapping positions

An F_2 population of 84 plants, derived from a cross between *L. esculentum* cv. Allround and *L. pennellii* LA716 (Haanstra et al. 1999b), was used to map 13 of the simple PCR markers (Fig.1, Table 2) that were polymorphic between these two parents. An RFLP map is available for this population, which contains a subset of RFLP markers used in the studies of Tanksley et al. (1992). By using the JoinMap 3.0 program (Van Ooijen and Voorrips, 2001), all the 13 simple PCR markers were integrated into this RFLP map, having map locations corresponding to their counterpart RFLP markers (data not shown). Generally, if one RFLP-probe has several copies in the genome, the derived PCR marker may detect only one of these copies. For example, the CAPS marker ct113 (Fig. 1) was mapped on Chromosome 7, while its counterpart RFLP probe (CT113) detects three copies on the tomato genome (on Chromosome 7, 10 and 11, respectively).

Strategies tried to streamline the conversion method

The method used in our study was an easy PCR-based approach, which can be easily applied to map ESTs (like U38666) that are potentially valuable sources of genetic markers and provide an opportunity to construct syntenic genome linkage maps of expressed genes among related species. In our study, we tried several strategies in order to streamline the method and to increase the efficiency for mapping ESTs. Firstly, for designing PCR primers from the cDNA clones, we initially tried to avoid an AGG triplet in primers, since an AGG triplet may be an exon junction site where intron RNA is removed and both adjacent exons are joined (Lang et al. 1998). Later, our results suggested that there was no strong association between the presence of an AGG triplet in the selected primers and the failure of PCR amplification (data not shown). Secondly, from the restriction analysis in this study, we learned that four or five base cutter enzymes were more efficient in detecting a single nucleotide polymorphism than restriction enzymes that have more selective nucleotides. Therefore, a CAPS-kit was assembled that contains 24 relatively cheap frequent-cutter restriction enzymes with different recognition sites (Table 3). In our laboratory, this CAPS-kit is being used efficiently and cost-effectively in the conversion of CAPS markers (Brugmans et al. 2003). Moreover, a short EST sequences could be elongated by performing a BLAST against TIGR database or GenBank database. For example, a BLAST against TIGR database was carried out for one RFLP probe CT218 (270 bp), an elongated gene sequence was selected to design a set of primers to amplify an intron that may exhibit more DNA polymorphism than exons (Cato et al. 2001, Temesgen et al. 2001). The derived CAPS marker mapped on Chromosome 3 in one F_2 mapping population, while the RFLP marker CT218 maps on Chromosome 9 and there are no other known mapped loci for this marker. Apparently, a different but partially homologous locus was amplified. Thus,

elongated gene sequences may be helpful to design PCR markers, but have the risk that other loci are amplified.

Table 3. List of the restriction enzymes in the CAPS-kit

Restriction enzyme	Sequence	Incubation temperature
Aci I	CCGC	37°C
Alu I	AGCT	37°C
Apo I	RAATTY	50°C
Bfa I	CTAG	37°C
*Bsa*J I	CCNNGG	60°C
*Bss*K I	CCNGG	60°C
*Bst*U I	CGCG	60°C
Dde I	CTNAG	37°C
Dpn I	GATC	37°C
Hae III	GGCC	37°C
Hha I	GCGC	37°C
Hinf I	GANTC	37°C
Hpa II	CCGG	37°C
*Hpy*188 I	TCNGA	37°C
*Hpy*CH4 III	ACNGT	37°C
*Hpy*CH4 IV	ACGT	37°C
Mnl I	CCTC	37°C
Mwo I	GCNNNNNNNGC	60°C
Nla III	CATG	37°C
Nla IV	GGNNCC	37°C
Rsa I	GTAC	37°C
*Sau*96 I	GGNCC	37°C
Taq I	TCGA	65°C
*Tsp*509 I	AATT	65°C

APPLICATION

The simple PCR markers presented in this study may have various applications for genetic studies and practical breeding programs in tomato. Firstly, as they are co-dominant markers with known map positions, they are very useful for integrating dominant markers like AFLPs in repulsion phase to improve linkage maps, and for assigning an unknown linkage group to a

chromosome (Bai et al 2003). Secondly, in practical breeding programs, they are useful diagnostic markers for marker assisted selection as they are less laborious and inexpensive. Moreover, they offer the advantage of a rapid transfer of linkage information among *Lycopersicon* species as they are locus specific. Some of these markers may serve as anchor points for comparative mapping between tomato and *Arabidopsis* (Ku et al. 2001, Fulton et al. 2002). In our research, some of these markers on Chromosome 12 (Table 2) have been successfully applied to localize *Ol-qtl2* and *Ol-qtl3*, conferring partial resistance to tomato powdery mildew disease. These markers not only allowed us to align genome linkage maps across distantly related species for revealing the co-localization between these QTLs and major genes, but also to monitor the presence of these QTLs in the development of near isogenic lines (Bai et al. 2003).

The detailed information of our CAPS/SCAR markers is maintained and updated regularly at URL: http://www.dpw.wau.nl/pv/CAPStomato/. We hope that our results will contribute to establish a tomato CAPS-marker database, similar to that for *Arabidopsis*: http://ausubellab.mgh.harvard.edu/resources/molecular/caps/.

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Chapter 4

Mapping *Ol-4*, a gene conferring resistance to *Oidium neolycopersici* and originating from *Lycopersicon peruvianum* LA2172, requires multi-allelic, single-locus markers

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ABSTRACT

Lycopersicon peruvianum LA2172 is completely resistant to *Oidium neolycopersici*, the causal agent of tomato powdery mildew. Despite the large genetic distance between the cultivated tomato and *L. peruvianum*, fertile F_1 hybrids of *L. esculentum* cv. Moneymaker x *L. peruvianum* LA2172 were produced and a pseudo-F_2 population was generated by mating F_1 half-sibs. The disease tests on the pseudo-F_2 population and two BC_1 families showed that the resistance in LA2172 is governed by one dominant gene, designated as *Ol-4*. In the pseudo-F_2 population, distorted segregation was observed and multi-allelic, single-locus markers were used to display different marker-allele configurations per locus. Parameters for both distortion and linkage between genetic loci were determined by maximum likelihood estimation and the necessity of using multi-allelic, single-locus markers was illustrated. Finally, a genetic linkage map of Chromosome 6 around the *Ol-4* locus was constructed by using the pseudo-F_2 population.

INTRODUCTION

Lycopersicon peruvianum is one of the wild relatives of tomato and provides a vast reservoir of valuable traits for crop improvement, such as disease, pest and virus resistances (e.g. Brüggemann et al. 1996, Ammiraju et al. 2003). *L. peruvianum* LA2172 is almost immune to *Oidium neolycopersici*, the causal agent of powdery mildew in tomato (Lindhout et al. 1994a). The resistance in *L. peruvianum* LA2172 is considered as complete, compared to the incomplete resistance conferred by the *Ol-1* and *Ol-3* genes that map on Chromosome 6 and originate from *L. hirsutum* G1.1560 and G1. 1290, respectively (Huang et al. 2000c, Van der Beek et al. 1994).

L. peruvianum is reproductively isolated from the "*esculentum* complex" by severe crossing barriers (Taylor 1986). These barriers can be partially overcome by using either *in vitro* techniques or bridge accessions like *L. peruvianum* LA1708 and LA2172 (Rick 1982; Poysa 1990; Van Heusden et al. 1999; Veremis and Roberts 1996).

L. peruvianum is an out-crossing species, with accessions and individuals within accessions differing in marker alleles at the same locus (Baudry et al. 2001; Ganal and Tanksley 1996; Van Ooijen et al. 1994). In several studies, where intraspecific crosses were used for mapping, multi-alleles at a single locus were encountered and different mapping strategies were used to deal with multi-allelic loci. One example is the study of Van Ooijen et al. (1994), in which a restriction fragment length polymorphism (RFLP) linkage map of *L. peruvianum* was constructed using three reciprocal backcross populations from an intraspecific cross between *L. peruvianum* LA2157 and LA2172. Multiple alleles were observed within the LA2172 accession, but only RFLP markers that yielded polymorphism between, but not within the two parents, were used. In this way, the multi-allelic loci were excluded. In contrary, in the study of Ganal and Tanksley (1996) multi-allelic loci were deliberately selected in order to estimate male and female recombination frequencies in F_1 mapping populations derived from different *L. peruvianum* plants. RFLP markers that simultaneously segregated in both male and female gametes (Ritter et al. 1990) were included and scored dominantly. Via this strategy, the number of useful RFLP markers was limited and the co-dominant information usually provided by RFLP markers was neglected.

Distorted marker segregation has been reported frequently in mapping studies of *L. peruvianum*, as is also reported in other inter- and intra-specific crosses within *Lycopersicon* (Kaloshian et al. 1998, Haanstra et al. 1999b, Sandbrink et al. 1995, Van Heusden et al. 1999, Zamir and Tadmor 1986). In *L. peruvianum*, this may be caused by both self-incompatibility and unilateral incompatibility, resulting in preferential transmission of certain alleles. One gametophytic self-incompatibility locus in *L. peruvianum* has been mapped on Chromosome 1 (Tanksley and Loaiza-Figueroa 1985). Three loci for the unilateral incompatibility in *L. pennellii* were identified on Chromosomes 1, 6 and 10 (Chetelat and De Verna 1991).

All of these phenomena, crossing barriers, multi-alleles per locus and distorted segregation hamper genetic studies of *L. peruvianum*. In this paper, we characterized the resistance to *O. neolycopersici* in *L. peruvianum* LA2172 by using different populations from an interspecific cross between *L. esculentum* cv. Moneymaker and *L. peruvianum* LA2172. Multi-allelic, single-locus markers were exploited to unfold the complexities such as multi-allelism and distorted segregation in a pseudo-F_2 population. We illustrated the risk of exclusive use of bi-allelic markers.

MATERIALS AND METHODS

Plant and fungal materials

Hundreds of pollinations were made on plants of *L. esculentum* cv. Moneymaker (MM) with pollen from two plants of *L. peruvianum* LA2172 (LA2172), and only 11 F_1 plants could be raised (Table 1). These F_1 plants were self-incompatible, so pseudo-F_2 populations were obtained by cross-pollinations between individual F_1 plants. Finally, a pseudo-F_2 population (hereafter referred as the F_2 population) of 194 plants, derived from a cross between two F_1 plants (F_{1a} and F_{1b}) that originated from different individual LA2172 pollen parents, was produced for the present study (Fig. 1). Different backcross populations were generated by using MM as a recurrent parent. Two BC_1 families (in total 80 plants) of the two F_1 plants (F_{1a} and F_{1b}) were used in the present study (Fig. 1).

Twenty informative F_2 plants of the *Ol-1* reference mapping population, which was derived from an interspecific cross of *L. esculentum* cv. Moneymaker x *L. hirsutum* G1.1560 (Fig. 3B, Huang et al. 2000b) were used to position molecular markers linked to the *O. neolycopersici* resistance in LA2172.

The pathogenic fungus *O. neolycopersici*, which originated from infected tomato plants (Lindhout et al. 1994a), was maintained on MM plants in a growth chamber at a temperature of 20 ± 2°C with 70% relative humidity (RH).

Disease test

The inoculum preparation and the inoculation were performed as described by Bai et al. (2003). The experimental setup was according to a randomized block design. For the disease test on the F_2 population, six blocks were used and each contained 32-33 F_2 plants, three LA2172 plants as resistant control and five MM plants as susceptible control. The inoculated plants were grown in a greenhouse at 20±3°C with 30-70% RH. The plant was scored as resistant (no visible fungal sporulation) or susceptible (with fungal sporulation) at 14, 17 and 21 days post

inoculation (dpi). For the disease tests on the BC_1, one block contained 80 BC_1 plants of the two BC_1 families, 20 plants of each MM and LA2172 as susceptible and resistant control, respectively. The inoculated plants grew in a greenhouse at 22°C with 70% RH, and were evaluated as described above at nine and 12 dpi.

Table 1. Generating of F_1 hybrids from crosses between *L. esculentum* cv. Moneymaker (MM) and *L. peruvianum* LA2172 (LA2172)

	MM x LA2172 (plant no. 1)	**MM x LA2172 (plant no. 2)**
Crosses	> 100	> 100
Fruits	17	20
Fruits with seeds	6	9
No. of seeds in total	22	29
No. of F_1 plants in total	9	2

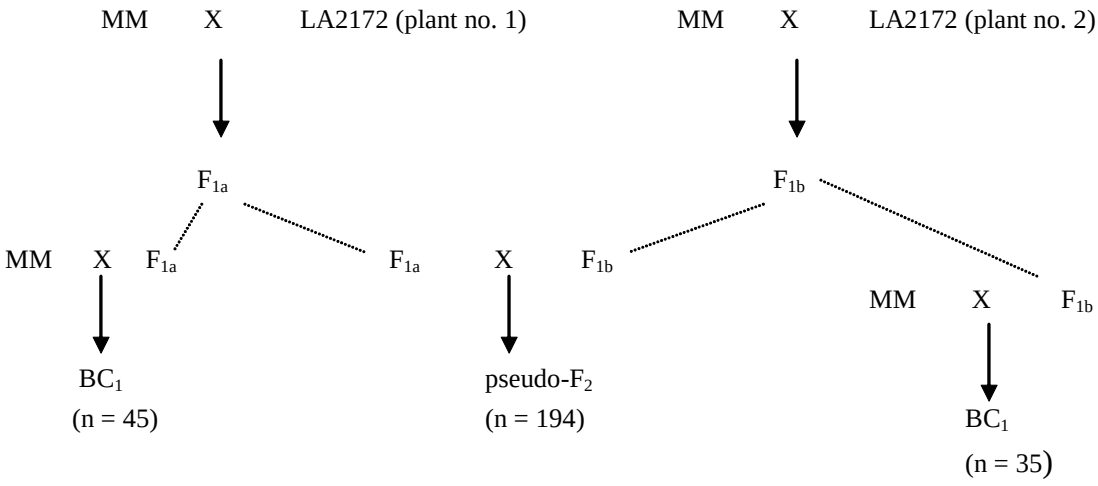


Fig. 1 Cross-pollinating scheme of BC populations and a pseudo-F_2 population from a cross of *L.esculentum* cv. Moneymaker (MM) x *L. peruvianum* LA2172 (LA2172).

Molecular markers and linkage analysis

As described by Bai et al. (2003), total DNA was extracted from leaves of the F_2 and BC_1 plants and the AFLP fingerprints were generated. Bulked segregant analysis (BSA) was performed for AFLP analysis on the resistant and susceptible pools, which were composed of equal volumes of AFLP pre-amplification products of eight resistant and eight susceptible F_2 plants, respectively. AFLP markers were converted into cleaved amplified polymorphic sequence (CAPS) markers according to Brugmans et al. (2003). The CAPS marker *Aps1* was generated as described by Bai et al. (2003).

The genetic linkage maps were constructed using the software package JOINMAP 3.0 (Van Ooijen and Voorrips 2001). A full account of linkage analysis using marker data of the F_2 population is given in the Appendix.

RESULTS

The resistance to O. neolycopersici in L. peruvianum LA2172

Fertile F_1 hybrids of MM x LA2172 were produced despite the large genetic distance between these two species. The F_1 progeny was not tested for resistance to *O. neolycopersici*, since it was very difficult to generate the F_1 plants and the development of these F_1 plants was very irregular. Instead, two BC_1 families from two F_1 plants (F_{1a} and F_{1b}) that were used for generating the F_2 population (Fig. 1), were analyzed for resistance to *O. neolycopersici*. As shown in Table 2, all plants of the susceptible control MM showed fungal sporulation and all LA2172 plants were immune (no symptoms). The BC_1 plants could unambiguously be classified as either resistant (R) or susceptible (S). One BC_1 family (MM x F_{1a}) was segregating for resistance (12 R : 33 S) (Table 2), suggesting that the ancestor F_{1a} plant was heterozygously resistant. And thus, resistance in LA2172 should be dominant although the segregation in this BC_1 family did not follow the 1 : 1 ratio of a monogenic model ($\chi^2 = 8.889$, P =0.003). All BC_1 plants (n = 35) of MM x F_{1b} were susceptible, indicating that the F_{1b} plant was homozygously susceptible. In the last 10 years, not a single LA2172 plant has been found to be susceptible to *O. neolycopersici*, despite extensive testing of this accession (data not shown). No susceptible LA2172 plants have been found so far; however a susceptibility allele was transferred to the F_{1b} plant, indicating that the resistance gene is heterozygously present in the LA2172 parent. To verify whether the resistance in LA2172 is monogenic, a large-scale disease test was performed on the F_2 population derived from the cross of F_{1a} x F_{1b}. As shown in Table 2, 100 F_2 plants were resistant and 94 were susceptible, which is in agreement with a segregation ratio of 1:1 for a

monogenic model (χ^2 = 0.1289, P = 0.72). We therefore concluded that the resistance in LA2172 is governed by one dominant gene, designated as *Ol-4*.

Table 2. Results of different disease tests on two BC_1 families and one pseudo-F_2 population from a cross of *L. esculentum* cv. Moneymaker (MM) x *L. peruvianum* LA2172 (LA2172)

Test	Plant	Number of plants		
		N (total)	R (resistant)	S (susceptible)
BC_1 families	MM	20	0	20
	LA2172	20	20	0
	BC_1 (MM x F_{1a})	45	12	33
	BC_1 (MM x F_{1b})	35	0	35
Pseudo-F_2 population	MM	30	0	30
	LA2172	18	18	0
	F_2	194	100	94

Identification of markers linked to the Ol-4 locus

Since the F_2 population was derived from a cross between two F_1 plants (F_{1a} x F_{1b}) that originated from different LA2172 plants (Fig. 1), it might harbor different marker alleles at one locus. Assuming this can also be true for markers linked to the *Ol-4* locus, the heterozygous resistant plant F_{1a} has one marker allele from LA2172 (coded as 'p', linked to the resistance allele of the *Ol-4* locus) and one marker allele from MM (coded as 'e'). The homozygous susceptible plant F_{1b} has one marker allele from LA2172 (coded as 'p*', linked to the susceptibility allele of the *Ol-4* locus) and one marker allele 'e' from MM. Therefore, four marker genotypes (p/p*, e/p, e/p* and e/e) segregating at 1:1:1:1 ratio were expected in the F_2 population. To test for this hypothesis, multi-allelic, single-locus markers were required to differentiate the four marker genotypes in the F_2 population.

In order to efficiently identify the marker allele p linked in coupling phase to the *Ol-4* locus, BSA was performed by using AFLP on the resistant and susceptible pools of the F_2 plants (see Materials and Methods). By using a total of 256 *Pst*/*Mse* primer combinations, 16 AFLP markers were identified that were present only in the resistant pool. Ten of these AFLP markers showed close co-segregation with *Ol-4* (Fig. 3A) by testing them on 56 F_2 individuals (20 resistant and 36 susceptible). To assign the AFLP markers to a particular chromosome, one AFLP marker (P18M51-450) was successfully converted into a CAPS marker, designated By-4 (Table 3). By

using the *Ol-1* reference mapping population (see Materials and methods), By-4 was positioned on Chromosome 6 between RFLP markers *Aps1* and TG153 (Fig. 3B).

Table 3. Primer sequences, lengths of PCR products and enzymes revealing a polymorphism for cleaved amplified polymorphic sequence (CAPS) markers

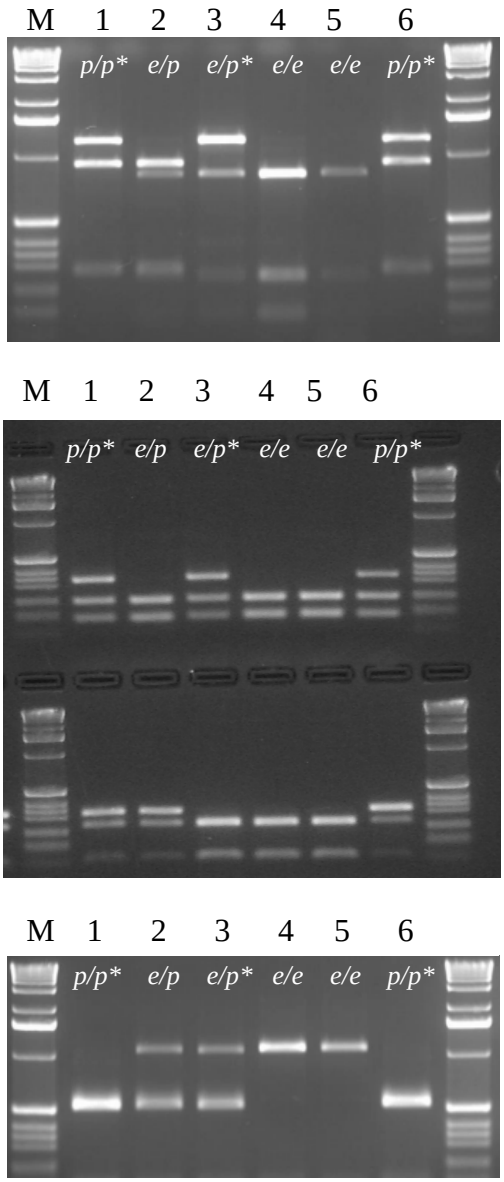
CAPS marker	Primer sequence (5'- 3')	Tm[a] (°C)	PCR products (bp)	Enzymes detecting polymorphism between	
				L. peruvianum **LA2172 and** ***L. esculentum*** **cv. Moneymaker**	***L. hirsutum*** **G1.1560 and** ***L. esculentum*** **cv. Moneymaker**
Aps1	F: atggtgggtccaggttataag	56	1300	*Sau*96 I ($p = p^* \neq e$)	*Dde* I
	R: cagaatgagcttctgccaatc			*Taq* I ($p \neq p^* \neq e$)	
By-4	F: catagtgtagctttgattcttgta	46	300	*Apo* I ($p \neq p^* = e$)	*Mse* I
	R: ccaattgccgggaaggaa			*Hyp*CH4 IV ($p = e \neq p^*$)	

[a] PCR annealing temperature.

Map position of the Ol-4 locus

In order to verify whether *Aps1* is linked to the *Ol-4* locus, the RFLP marker *Aps1* on Chromosome 6 was converted into a CAPS marker (Table 3) that co-segregated with the original RFLP marker in the *Ol-1* reference mapping population (see Materials and Method). The CAPS marker *Aps1/Taq*I revealed the expected multiple alleles in the F_2 population. As shown in Fig. 2A, the e allele was from MM, two other alleles (p and p*) were from LA2172. By testing this marker on the F_2 population, four marker phenotypes were observed (p/p*, e/p, e/p* and e/e, Table 4). Only the marker allele p was linked to the resistance allele of the *Ol-4* locus. In addition, for the CAPS marker By-4 (Fig. 2B, Table 4), restriction enzyme *Hyp*CH4IV was specific for the p* allele (in equation $p^* \neq p = e$), and restriction enzyme *Apo*I was specific for the p allele (in equation $p \neq p^* = e$). Thus, when the results from these two restriction enzymes were combined, marker By-4 uncovered the four distinct marker genotypes of the F_2 plants. The three marker alleles for CAPS markers *Aps1* and By-4 were confirmed in the two BC_1 families. The expected ratio for the four marker genotypes (p/p*, e/p, e/p* and e/e) in the F_2 population was 1:1:1:1, while the observed frequencies were 74 : 15 : 80 : 17 for marker *Aps1*, and 73 : 16 : 80 :17 for marker By-4, respectively (Table 4). For both CAPS markers By-4 and *Aps1*, distorted marker segregation was caused by preferential transmission of the *L. peruvianum* allele by the male parent (genotype

e/p*, Appendix C), with estimated transmission rates of about 18% and 82% for allele e and p*, respectively.



(A) CAPS marker *Aps1/Taq* I ($p \neq p^* \neq e$). The upper fragment in lane 6 (LA2172) is the p^* allele and the lower fragment is the *p* allele; the single fragment in lane 5 (MM) is the *e* allele.

(B) CAPS marker By-4. In lane 6 (LA2172) of the upper panel, *Hpy*CH4 IV ($p = e \neq p^*$) reveals the upper fragment for p^* allele; in lane 6 (LA2172) of the lower panel, *Apo* I ($p \neq p^* = e$) yields the upper fragment for *p* allele.

(C) CAPS marker *Aps1/Sau*96 I ($p = p^* \neq e$). The fragment in lane 6 (LA2172) is diagnotic for both *p* and p^* alleles.

Fig. 2 Electrophoretic patterns of CAPS markers (2% agarose gel) to show marker phenotypes in a pseudo-F_2 population from a cross of *L.esculentum* cv. Moneymaker (MM) x *L. peruvianum* LA2172 (LA2172). M indicates DNA size marker of 1kb ladder. Lane 1 and 2 are resistant F_2 plants, lane 3 and 4 are susceptible F_2 plants. Lane 1 to 4 show marker phenotypes of *p/p**, *e/p*, *e/p** and *e/e*, respectively. Lane 5 is MM with marker phenotype of *e/e*, and lane 6 is LA2172 (DNA is pooled from several plants) ans shows marker phenotype of *p/p**.

Table 4. Marker genotypes in a pseudo-F_2 population derived from a cross of *L. esculentum* cv. Moneymaker x *L. peruvianum* LA2172

Marker genotype	**Expect-ed ratio**	**Number of the F_2 plants per marker phenotype**				
		Aps1*/*Taq* I** (p* ≠ *p** ≠ *e*)	***Aps1*/*Sau*96 I** (***p* = *p** ≠ *e*)	**By-4 (*Apo* I + *Hyp*CH4 IV)** (***p* ≠ *p** ≠ *e*)	**By-4/*Apo* I** (***p* ≠ *p* * = *e*)	**By-4/*Hyp*CH4 IV** (***p* = *e* ≠ *p* ***)
*p/p**	1:4	74 (71 R + 3 S)[a]	74 (71 R + 3 S)	73 (70 R + 3 S)	89 (85 R + 4 S) (*e/p* = *p/p**)	153 (78 R + 75 S) (*p/p** = *e/p**)
e/p	1:4	15 (15 R)	95 (22 R + 73 S) (*e/p* = *e/p**)	16 (15 R + 1 S)	See *p/p**	33 (15 R + 18 S) (*e/p* = *e/e*)
*e/p**	1:4	80 (7 R + 73 S)	See *e/p*	80 (8R + 72 S)	97 (8 R + 89 S) (*e/p** = *e/e*)	See *p/p**
e/e	1:4	17 (17 S)	17 (17 S)	17 (17 S)	See *e/p**	See *e/p*

[a] R: resistance; S: susceptible

All possible marker-allele configurations in the F_2 population are shown in Table 4 and Fig. 2, and marker data for cross pollinator (CP) population type were prepared for JoinMap to construct a linkage map around the *Ol-4* locus. As the JoinMap program assumes no distortion, parameters for both distortion and linkage between the *Ol-4* and CAPS markers were also estimated using maximum likelihood estimations. A full account of the linkage analysis for various types of the CAPS markers is given in the Appendix. A linkage map was constructed for a part of tomato Chromosome 6, where the *Ol-4* locus was positioned above CAPS marker *Aps1* (Fig. 3A). A comparison of the *Ol-4* maps with the *Ol-1* map showed that the order of the marker loci on the maps was identical. Therefore, we concluded that *Ol-4* is on Chromosome 6, but at a position different from the *Ol-1* locus.

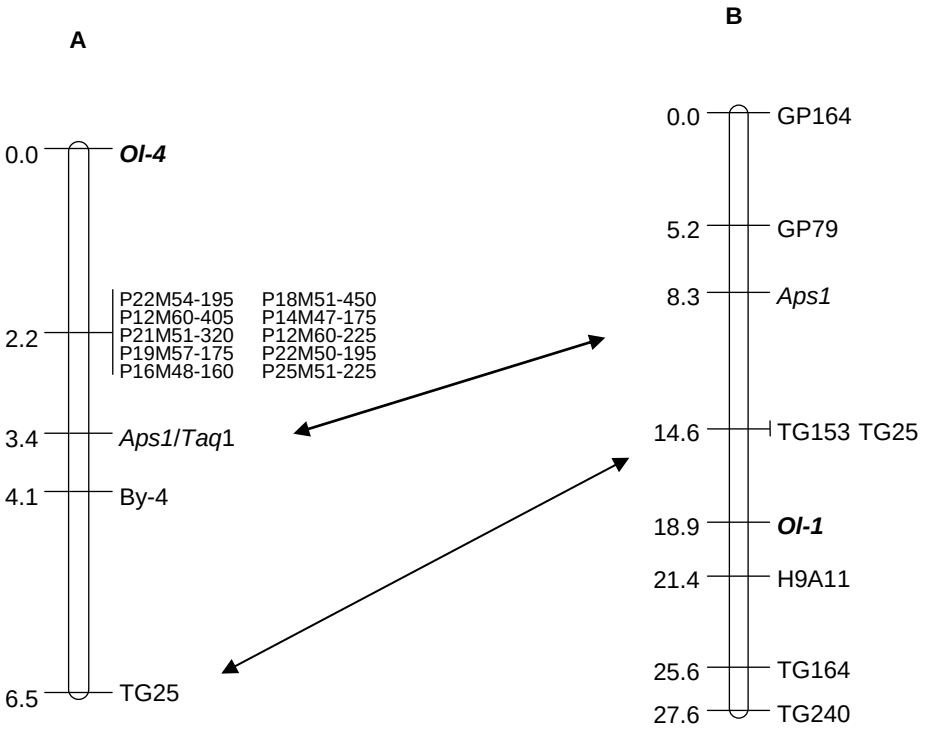


Fig. 3 Genetic maps of part of Chromosome 6 showing map positions of *Ol-4* and *Ol-1* loci based on two different populations. (A), the *Ol-4* locus map position in a pseudo-F_2 population derived from the cross of *L. esculentum* cv. Moneymaker x *L. peruvianum* LA2172. AFLP and CAPS markers were used. (B), skeleton map showing positions of the *Ol-1* locus and some RFLP markers on Chromosome 6 that was retrieved from Huang et al. (2000b).

DISCUSSION

L. peruvianum is an out-crossing species and can harbor multi-alleles per locus as illustrated in the present study and by others (Ganal and Tanksley 1996, Kaloshian et al. 1998, Miller and Tanksley 1990, Van Ooijen et al. 1994). In several mapping studies, mapping strategies have been applied in order to circumvent the complexities of multi-allelism (Van Ooijen et al. 1994, Ganal and Tanksley 1996). In the present study, it was illustrated that the information of multi-alleles at one locus can be fully exploited. Taking into account the high degree of heterogeneity in the donor parent *L. peruvianum* LA2172, we anticipated the possibility of more than two marker alleles per locus in the pseudo-F_2 population that was generated by mating F_1 half-sibs. To test this hypothesis, multi-allelic CAPS markers for single loci were developed, which uncovered all marker-allele configurations (four marker genotypes, Fig. 2) in the F_2 population. Consequently, markers could not be processed in the same way as a normal backcross or F_2 population from inbreeding species, but rather as a population resulting from a cross between

two heterogeneously heterozygous diploid parents (coded as CP, cross pollinator, in JoinMap program).

The recognition of multi-alleles at a single locus in the F_2 population by the multi-allelic markers has been of crucial importance. As shown in Table 4, CAPS markers uncovered two, three or four genotypes in the F_2 population depending on the restriction enzymes used (Fig. 2). For example, the CAPS marker *Aps1*/*Sau*96I could not differentiate between the alternative *L. peruvianum* alleles p and p* (Fig. 2C). Thus, this marker revealed only three marker phenotypes in the F_2 population: homozygous as LA2172 (p/p*), homozygous as MM (e/e) and heterozygous (marker genotype of e/p and e/p*). F_2 plants carrying the heterozygous marker phenotype segregated into classes: resistant (n = 22) and susceptible (n = 73) (Table 4). As explained in Appendix D, incorrect estimates of recombination frequency between the *Ol-4* locus and this marker could be obtained, if the distortion cannot unambiguously be attributed to either of the parents. Thus, the usage of bi-allelic markers of this type may complicate the mapping study because of the joint effect of linkage and preferential transmission of certain alleles. Similarly, bi-allelic markers like CAPS marker By-4/ *Hyp*CH4IV (where p = e $\neq$ p*) were not informative for mapping in this pseudo-F_2 population (Appendix A), as this marker type uncovers two marker phenotypes (Fig. 2B and Table 4). One is heterozygous as LA2172 (marker genotypes of p/p* and e/p*) with 153 F_2 plants (78 resistant and 75 susceptible), while the other is homozygous as MM (marker genotypes of e/p and e/e) with 33 F_2 plants (15 resistant and 18 susceptible). Without discrimination of the p and e alleles one would conclude that By-4/ *Hyp*CH4IV segregates independently from the *Ol-4* gene. For a reliable genetic analysis, it is crucial to have markers that reveal different marker-allele configurations, like the multi-allelic single locus marker *Aps1*/*Taq*I that detected the three marker alleles (p, p* and e) and distinguished the four marker genotypes in the F_2 population (Fig. 2A and Table 4).

By recognition of multi-alleles at a single locus in the F_2 population, distorted segregation in the F_2 population became uncovered that was caused by preferential transmission of *L. peruvianum* alleles by the male parent only. In this case, it was demonstrated that the selection acted against male gametes having *L. esculentum* alleles at the *Ol-4* locus, which hardly influences the estimated recombination frequencies between the genetic loci (Appendix C). In agreement with this, other authors (Ritter et al. 1990, Van Ooijen et al. 1994) suggested that the estimated recombination frequency is unbiased when the segregation distortion is caused by selection at one locus per chromosome, but is biased when the selection acts on two (or more) loci on one chromosome.

In summary, we mapped the *Ol-4* gene on the tomato Chromosome 6, which governs complete resistance to *O. neolycopersici* and originates from *L. peruvianum* LA2172. It was illustrated that the hypothesis of close linkage between genetic markers can be erroneously

rejected in case bi-allelic marker data are used for multi-allelic loci. The recognition of multiple alleles allowed accurate mapping of *Ol-4* and revealed the large segregation distortion in the pseudo-F_2 population of MM x LA2172.

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Chapter 5

Three loci on tomato Chromosome 6 containing genes (*Ol*-genes) conferring resistance to *Oidium neolycopersici*

Submitted.

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ABSTRACT

In plants, resistance genes occur in a number of different genomic arrangements. Frequently, they are members of multigene families that are located in tandem arrays and form clusters. Our research aims at studying the dispersion of *Ol*-genes conferring resistance to *Oidium neolycopersici* across the *Lycopersicon* genome. In this study, an experimental approach has been chosen to use a set of common locus specific PCR markers in different mapping populations segregating for *Ol*-genes. This approach allowed us to generate an integrated map comprising DNA markers and different *Ol*-genes. Remarkably, all the dominant *Ol*-genes mapped so far are located on tomato Chromosome 6 and are organized in three loci. On the long arm, one locus contains two genes *Ol-1* and *Ol-3* (originating from *L. hirsutum* G1.1560 and G1.1290, respectively) and the other locus harbors *Ol-5* (originating from *L. hirsutum* PI247087). Only two markers were found to separate these two loci at a genetic distance of about 1 cM. On the short arm, one locus contains two genes *Ol-4* (originating from *L. peruvianum* LA2172) and *Ol-6* (unknown origin). Although the map positions of *Ol-4* and *Ol-6* were identical, these two genes appeared to have a different model of action in response to fungal infection.

INTRODUCTION

In plants, resistance genes occur in a number of different genomic arrangements. The simplest arrangement is a locus consisting of a single resistance gene, which can show considerable allelic genetic variation. The best characterized gene of this type is the *L* gene of flax, where multiple alleles have been identified by their differential reactions to flax rust (*Melampsora lini*) races (Islam and Shepherd 1991). More frequently, resistance genes are members of multigene families and located in tandem arrays, forming clusters (Michelmore and Meyers 1998). It can be a simple cluster with narrowly spaced homologous genes, like the *Mi*, *I-2* and *Cf* gene clusters in tomato and the *Xa21* gene cluster in rice (Dixon et al. 1996, Ori et al. 1997, Parniske et al. 1999, Seah et al. 2004, Song et al. 1997). It can also be a more complex cluster, where homologous genes are widely dispersed over a chromosome arm like the *Dm3* cluster in lettuce where twenty-four homologues of *Dm3* gene are dispersed over the genetic region surrounding *Dm3* spanning at least 3.5 Mb (Meyers et al. 1998). Clusters of homologous genes conferring specific resistances to different races of a pathogen can be closely linked, and may have evolved from common ancestors by local gene duplications followed by structural and functional diversification (Gebhardt and Valkonen 2001). One very well characterized example of this arrangement is the *Hcr9* gene family, harboring homologues of the *Cladosporium fulvum* resistance gene *Cf-9*. Five linked clusters of *Hcr9s* have been identified, distributed over about 40 cM on the short arm of tomato Chromosome 1, three of which contain functional *Cf*-genes that differ in specificity (Haanstra et al. 1999a, Parniske et al. 1999). Interestingly, clusters of homologous genes conferring resistances to different pathogens may also be linked (Grube et al. 2000, Hulbert et al. 2001). For example, the *Mi*-cluster is linked to the *Cf-2*-cluster on the short arm of tomato Chromosome 6. The *Mi* gene confers resistances against three very different organisms including root-knot nematodes, aphids and whitefly (Nombela et al. 2003), while the *Cf-2* gene confers resistance to *Cladosporium fulvum* (Dickinson et al. 1993).

Our research aims at studying the distribution of resistance genes to tomato powdery mildew (*Oidium neolycopersici*) over the *Lycopersicon* genome. The cultivated tomato is susceptible to the fungus, but resistance occurs in many wild *Lycopersicon* species (Lindhout et al. 1994a). The dominant genes *Ol-1* and *Ol-3*, which confer incomplete resistance to *O. neolycopersici* and originate from *L. hirsutum* G1.1560 and G1.1290, respectively, have been mapped on the long arm of tomato Chromosome 6 (Lindhout et al. 1994b, Huang et al. 2000b&c). Another dominant gene *Ol-4* that confers complete resistance to *O. neolycopersici* and originates from *L. peruvianum* LA2172 is also positioned on Chromosome 6 but at a position different from the *Ol-1*/*Ol-3* locus (Bai et al. 2004b). Moreover, three quantitative trait loci (QTLs) have been identified governing the resistance to *O. neolycopersici* in *L. parviflorum* G1. 1601. One QTL (*Ol-qtl1*) interval overlaps with *Ol-1* and *Ol-3*, and two other QTLs (*Ol-*

qtl2 and *Ol-qtl3*) are linked on Chromosome 12 in the vicinity of the *Lv* gene, conferring resistance to another powdery mildew species, *Leveillula taurica* (Bai et al. 2003). In addition, a recessive resistance gene (*ol-2*) has been reported in *Lycopersicon esculentum* var *cerasiforme* (Ciccarese et al. 1998), which is located on Chromosome 4 (De Giovanni et al. 2004). Finally, a very high level of resistance to *O. neolycopersici* was identified in *L. hirsutum* PI247087 (Latterot et al. 1995), and the preliminary results showed that this resistance was probably polygenic, while the locus with the major effect on resistance was probably linked to the gene *Ol-1* (Moretti and Caranta 2000).

In this study, we aimed at characterizing the genome organization of *Ol*-genes on tomato Chromosome 6 in more detail. Therefore, we fine-mapped *Ol-1* and *Ol-4*. In addition, we characterized the resistance originating from *L. hirsutum* PI247087 (named *Ol-5*) and a newly identified resistance from an advanced breeding line (named *Ol-6*). As we focused on finding the accurate relative positions of the *Ol*-genes on a molecular map, we have chosen an experimental approach to use a set of common locus specific PCR markers in different mapping populations segregating for *Ol*-genes. This approach allowed us to generate an integrated map comprising DNA markers and different *Ol*-genes. Our data showed that the five dominant *Ol*-genes mapped so far are all located on tomato Chromosome 6 and organized in three genetic loci. Such an organization mirrors the arrangement of *Cf* genes on Chromosome 1. The relevance with respect to genome organization of the *Ol*-genes and their race-specificity are discussed.

MATERIALS AND METHODS

Plant materials

The plant materials used in this study are presented in Table 1. All the backcross populations were generated by using *L. esculentum* cv. Moneymaker (MM) as the recurrent parent.

Fungal material

The pathogenic fungus *O. neolycopersici*, which originated from infected tomato plants (Lindhout et al. 1994a), was maintained on MM plants in a growth chamber at 20±2°C, 70% relative humidity (RH) and a photoperiod of 16 hours.

Disease test

Detailed information for disease tests is presented in Table 2. The inoculum preparation and the inoculation were performed as described by Bai et al. (2003). The inoculated plants were grown in a greenhouse at 21°C with 70% RH under natural light supplemented with artificial light to

provide a photoperiod of 16 hours. The experiments were carried out according to a completely randomized design. The inoculated plants were evaluated in two ways. For complete resistance conferred by *Ol-4* or *Ol-6*, inoculated plants were scored as resistant (no visible fungal sporulation) or susceptible (with fungal sporulation). For incomplete resistance conferred by *Ol-1* or *Ol-5*, inoculated plants were evaluated according to the following disease index (DI), 0 = no visible sporulation, 1 = very few fungal spots surrounded by necrosis (weak sporulation), 2 = moderate number of fungal spot (moderate sporulation), 3 = very high number of fungal spots (heavy sporulation). An average DI was calculated over two times of evaluation (Table 2) for each plant. Plants were considered as resistant when DI ≤1, as susceptible when DI >2 and as incompletely resistant when 1<DI≤2. The resistance of the plants with 1<DI≤2 was re-evaluated by performing disease tests on their selfed progenies as described above.

Table 1. Plant materials

***Ol*-genes**	**Purpose**	**Mapping population**	**Original cross**	**Reference**
Ol-1	Bulked segregant analysis (BSA)	F_2	*L. esculentum* cv. Moneymaker (MM) x *L. hirsutum* G1.1560	This F_2 population was tested for resistance to *O. neolycopersici* and screened for 15 RFLP markers specific for Chromosome 6 and four SCAR markers (Huang et al. 2000b).
	To position markers linked to *Ol-1*	F_2 recombinants[a]		
	Recombinant screening	BC_1S_1	MM x ABL-*Ol-1* (ABL, advance breeding line)	ABL-*Ol-1* contains the *Ol-1* gene from *L. hirsutum* G1.1560 (Syngenta, Enkhuizen, The Netherlands).
Ol-4	Fine-mapping	BC_2S_1	MM x *L. peruvianum* LA2172	Bai et al. 2004b
Ol-5	Inheritance study and mapping	BC_2 and BC_2S_1	MM x ABL_1	ABL_1 was obtained from INRA (Montfavet, France). The resistance is derived from *L. hirsutum* PI247087.
Ol-6	Inheritance study and mapping	BC_2 and BC_3S_1	MM x ABL_2	ABL_2 was obtained from a breeding company. The origin of the resistance is unknown.

[a] A set of 16 informative genotypes is selected as a test-panel. These are recombinants in the interval between RFLP markers TG153 and TG164 and provide conclusive evidence for markers inside the interval between markers TG25 and H9A11 (Fig. 1A).

Table 2. Summary of the experiments with disease tests

Ol-genes	Aim of the test	Population(s)	Susceptible and resistant control	Evaluation
Ol-1	Fine-mapping	17 recombinant inbred lines (BC_1S_2), 16 plants per line	20 MM plants and 20 ABL-*Ol-1* plants	Using DI (disease index) of 0 to 3 at 11 and 13 dpi[a].
Ol-4	Fine-mapping	BC_2S_1 (n = 112)	20 MM and 20 LA2172 plants	Scoring plants as R[b] or S at 9 and 12 dpi.
Ol-5	Inheritance study	Three BC_2 (n = 79, in total)	20 MM plants and 20 ABL_1 plants	Using DI at 11 and 13 dpi.
Ol-5	Mapping	BC_2S_1 (n = 100)	20 MM and 20 ABL_1 plants	Using DI at 11 and 13 dpi.
Ol-6	Inheritance study	Two BC_2 (n = 52, in total)	20 MM plants and 20 LA2172 plants	Scoring plants as R or S at 11 and 13 dpi.
Ol-6	Mapping	BC_3S_1 (n = 80)	10 MM plants and 10 LA2172 plants	Scoring plants as R or S at 13 and 15 dpi.

[a] dpi, days post inoculation; [b] R = resistant and S = susceptible.

Marker analysis

Total DNA was extracted from leaves of the plants prior to inoculation by using a rapid CTAB DNA isolation method as described by Brugmans et al. (2003) and the AFLP fingerprints were generated on a LI-COR 4200 DNA sequencer as described by Bai et al. (2003). BSA was performed on resistant and susceptible pools that were composed of equal volumes of AFLP pre-amplification products of resistant and susceptible plants. AFLP markers were converted into simple PCR markers, such as CAPS (cleaved amplified polymorphic sequence) and SCAR (sequence characterized amplified region) according to Brugmans et al. (2003). RFLP markers (Tanksley et al. 1992) were converted to simple PCR markers as described by Bai et al. (2003). Primers for CAPS markers GP79L and 32.5Cla have been published by Kaloshian et al. (1998) and Liharska (1998), respectively. Detailed information of the PCR markers used in this study is presented in Table 3.

Linkage analysis

The genetic linkage maps were constructed by using the software package JOINMAP 3.0 (Van Ooijen and Voorrips 2001).

RESULTS

Fine-mapping of Ol-1

Previously, the gene *Ol-1* has been mapped on the long arm of tomato Chromosome 6 by using an F_2 population of MM x *L. hirsutum* G1.1560 (Huang et al. 2000b). In order to identify markers that are more closely linked to *Ol-1*, eight resistant (R) and eight susceptible (S) F_2 plants were selected from this F_2 population to construct R and S pools for bulked segregant analysis. The R pool was homozygous for *L. hirsutum* alleles and the S pool homozygous for *L. esculentum* alleles in an interval including the *Ol-1* locus between RFLP markers TG153 and TG164 (Fig.1A). By using 400 *Pst/Mse* AFLP primer combinations, 62 candidate AFLP markers were identified. By using a test-panel of 16 informative F_2 recombinants (Table 1&2), 17 of the 62 markers mapped between RFLP markers TG25 and H9A11 (an interval including the *Ol-1* locus). In this interval, two AFLP markers, M1349 and M2147, were most closely linked to *Ol-1*.

To facilitate recombinant screening in the region flanked by TG25 and H9A11, RFLP probes (TG25 and H9A11) and the two AFLP markers (M1349 and M2147) were sequenced and converted into simple PCR markers (Table 3). One BC_1S_1 population of 1413 plants derived from the cross of MM x ABL-*Ol-1* (Table 1) was screened for recombinants between PCR markers tg25 and H9A11. This provided 17 recombinants. The genetic distance between these two markers was estimated to be 0.64 cM in this population, which is approximately ten times lower than the distance in the F_2 population from an interspecific cross (Fig. 1A, Huang et al. 2000b). The 17 BC_1S_1 recombinants were selfed to produce BC_1S_2 lines that were subsequently tested for disease resistance and screened for markers in the region flanked by TG25 and H9A11. One recombinant (1/17) showed that *Ol-1* is below CAPS marker M1349. No recombinants were found between *Ol-1* and CAPS marker H9A11, indicating that H9A11 remained the closest linked marker below *Ol-1* (Huang et al. 2000b). By using a test-panel of 16 informative F_2 recombinants (Table 1), two recombinants showed that M1349 is below SCARF10, the closely linked marker above *Ol-1* as reported by Huang et al. (2000b, Fig.1A). Thus, *Ol-1* is fine-mapped between CAPS markers M1349 and H9A11 on the long arm of tomato Chromosome 6 (Fig. 1B). As the recombinants were obtained from different 'source populations' (the ABL-*Ol-1* derived BC_1S_1 population and the interspecific F_2 population, Table 1) with seriously different recombination frequencies, it was difficult to assess the genetic distance between *Ol-1* and the closely linked markers. On the basis of the ABL-*Ol-1* derived populations alone it was 0.035 cM (1/2826 gametes) between *Ol-1* and CAPS marker M1349, while in the interspecific F_2 population, this distance was ten times higher (about 0.35 cM).

Table 3. Primer sequences, lengths of PCR products and enzymes revealing a polymorphism for CAPS/SCAR markers [a]

Marker name	[b]Marker type	Primer name	Primer sequence (5'- 3') [c]	[d]Tm (°C)	PCR product size (bp)	Restriction enzymes detecting polymorphism between *L. esculentum* cv. Moneymaker and				Original marker type
						L. hirsutum G1.1560	*L. peruvianum* LA2172	ABL_1	ABL_2	
dM1346	dCAPS	dM1346-F dM1346-R	ttggaatcaagaaaaggggttaa atgactatgtccataaatgaa	47	200	[e] nd	*Hin*c II	nd	*Hin*c II	AFLP
GP79L	CAPS	GP79L-F GP79L-R	cactcaatgggggaagcaac aatggtaaacgagcgggact	56	2000	*Apo* I	*Apo* I	*Apo* I	*Apo* I	RFLP
32.5Cla	CAPS	32.5Cla-F 32.5Cla-R	acacgaaacaaagtgccaag ccaccaccaaacaggagtgtg	56	773	*Hin*f I	*Hin*f I	nd	*Hin*f I	RFLP
Aps1	CAPS	*Aps1*-F *Aps1*-R	atggtgggtccaggttataag cagaatgagcttctgccaatc	56	1000	*Dde* I	*Sau*96 I	*Hin*f I	nd	RFLP
M2147	CAPS	M2147-F1 M2147-R1	taacaatctcgaccatagttcc ccatacccgaatttccttcc	56	300	*Dde* I	*Dde* I	*Hae* III	nd	AFLP
M1349	CAPS	M1349-F M1439-R	tgctaagaatcagaaaccacacct acaacaagctgatccacctaaaga	56	500	*Xcm* I	nd	*Xcm* I	nd	AFLP
ct184	CAPS	Ct184-F Ct184-R	tttccgtgtattgccaacaa accaaagagtcaatggatgg	56	298	*Dde I*	*Dde* I	*Dde* I	nd	RFLP
tg25	Co-dominant SCAR	tg25-conF tg25-prR1	taatttggcactgccgt ttgtyatrttgtgyttatcg	52	350+300	No SCAR	SCAR	SCAR	nd	RFLP
tg25	Co-dominant SCAR	tg25-conF tg25-ehR1	taatttggcactgccgt catgttgygyttatcraaagtc	52	350+300	SCAR	No SCAR	SCAR	nd	RFLP
H9A11	Co-dominant SCAR	H9A11-conF H9A11-conR	tgctctaacaaaatcaccaaaatc aaatggtcaaacaaagtctattgag	52	450+400	SCAR	SCAR	SCAR	nd	RFLP
ct174	CAPS	ct174-F2 ct174-R1	aaaaagaagccccaaatagacc ggtcctccccatcaacagtc	56	500	*Hae* III	*Hae* III	*Hae* III	nd	RFLP

[a] The information will be maintained and updated regularly at: http://www.dpw.wau.nl/pv/CAPStomato/

[b] CAPS, cleaved amplified polymorphic sequence; dCAPS, derived CAPS; SCAR, sequence characterized amplified region.

[c] Codes for the degenerated position of primer tg25 are: Y, C/T; R, A/G.

[d] PCR annealing temperature.

[e] nd, not subjected to restriction enzyme screening.

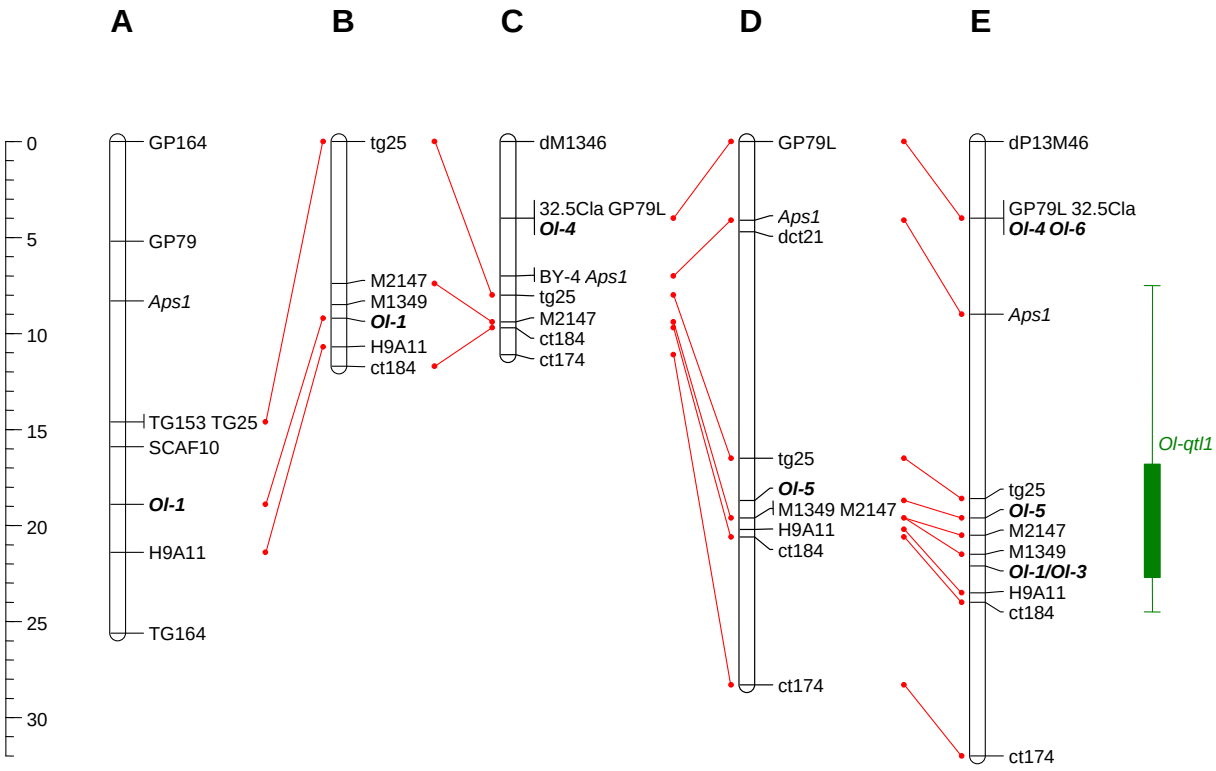

Fig. 1. Genetic maps for part of Chromosome 6 showing map positions of *Ol*-genes based on different populations. **A,** Skeleton map of *Ol-1* showing map positions of the RFLP markers and a SCAR marker (SCAF10) that are linked to *Ol-1*, which are retrieved from Huang et al. (2000b). **B,** *Ol-1* fine-mapping position, using a BC_1S_1 population of *L. esculentum* cv. Moneymaker (MM) x ABL-*Ol-1*. Based on the data set of the recombinant screening, relative orders for marker loci are indicated on this map rather than absolute map distances. **C,** Genetic linkage map showing map position of *Ol*-4 based on a BC_2S_1 population derived from a cross of MM x *L. peruvianum* LA2172. **D,** Genetic linkage map showing the map position of *Ol-5* based on a BC_2S_1 population derived from a cross of MM x ABL_1. **E,** Skeleton maps for tomato Chromosomes 6, showing relative positions of the mapped *Ol*-genes and *Ol-qtl1* (Bai et al. 2003). Bars indicate the QTL intervals for which the inner bar shows a one-LOD support interval and the outer bar shows a two-LOD support interval. Simple PCR markers are used for Fig. 1B to 1E.

Fine-mapping of Ol-4

Ol-4 has previously been mapped above CAPS marker *Aps1* on tomato Chromosome 6 by using a pseudo-F_2 population of MM x *L. peruvianum* LA2172 (Bai et al. 2004b). Further fine-mapping of *Ol-4* in this pseudo-F_2 population was complicated as multi-alleles at a single locus were encountered. To reduce the number of segregating alleles at the same locus, a BC_2S_1 (n = 112, Table 1) population of MM x LA2172 was generated and subjected to a disease test. This

population segregated for resistance to *O. neolycopersici* in a ratio of 77R: 35S, in accordance with the 3 : 1 ratio of a monogenic model (χ^2 = 2.012, P = 0.16). Chromosome 6 specific PCR markers (Table 3) were scored in this BC_2S_1 population. A linkage map was constructed for part of tomato Chromosome 6, and no recombinants were found between *Ol-4* and PCR markers GP79L and 32.5Cla (Fig. 1C). This linkage was confirmed in BC_3S_1 populations of MM x LA2172 (96 BC_3S_1 plants in total). On the short arm of tomato Chromosome 6, two loci show homology to marker GP79L (Kaloshian et al. 1998). One is within an introgressed region from *L. peruvianum* of approximately 650 kb containing the *Mi* gene, the other is above this region and closer to the telomeric end. The 32.5Cla locus maps close to the distal end of the 650 kb *Mi-1* introgressed region on the short arm of tomato Chromosome 6 (Liharska 1998). As the *Ol-4* locus was completely linked to GP79L and 32.5Cla, the map position of the *Ol-4* gene is likely close to the centromere on the short arm of Chromosome 6 (Fig. 1C).

Mapping of Ol-resistance originating from L. hirsutum PI247087

One advanced breeding line (ABL_1), obtained from INRA (Montfavet, France), contains resistance to *O. neolycopersici* introgressed from *L. hirsutum* PI247087. A preliminary study of Moretti and Caranta (2000) indicated that the resistance in *L. hirsutum* PI247087 was polygenic as well as closely linked to *Ol-1*, implying that a major gene near *Ol-1* and one or more QTLs contribute to this resistance. Our aim was to determine the inheritance of this resistance and map the major resistance. The inheritance of the resistance was studied by applying a disease test on three BC_2 families of the cross MM x ABL_1 (Table 1). The inoculated plants were evaluated for resistance using a disease index DI scale of 0 to 3 (see Materials and Methods). The segregation of resistant to susceptible plants in each BC_2 family, as well as jointly, fitted a segregation ratio of 1 R : 1 S for a monogenic dominant trait (Table 4), suggesting that one dominant gene (designated as *Ol-5*) governed the resistance in ABL_1. To map *Ol-5*, a set of co-dominant PCR markers on tomato Chromosome 6 (Table 3) were scored in one BC_2S_1 population (n = 100, Table 1). Especially, the PCR markers closely linked to *Ol-1* were selected in order to compare the map positions between *Ol-1* and *Ol-5*. A genetic linkage map was constructed for *Ol-5* that was positioned between PCR markers tg25 and M2147 on tomato Chromosome 6 (Fig. 1D). A comparison of the *Ol-5* map with the *Ol-1* map showed that orders of the marker loci on both maps were identical. As *Ol-5* maps proximal to the PCR marker M2147, it is likely that *Ol-5* represents another *Ol*-locus on the long arm of tomato Chromosome 6, about 1 cM proximal of the locus containing the *Ol-1* gene (Fig. 1E).

Table 4. Results of different disease tests on different BC_2 families derived from crosses between *L. esculentum* cv. Moneymaker (MM) x ABL_1 or ABL_2

***Ol* genes**	**Cross**	**BC_2 families**	**Plant number**			
			Resistant	Susceptible	χ^2	P
Ol-5	MM x ABL_1	BC_{2a} (n = 30)	13	17	0.83	0.36
		BC_{2b} (n = 30)	15	15	0.03	0.86
		BC_{2c} (n = 19)	11	8	0.21	0.65
		Total (n = 79)	39	40	0.00	
Ol-6	MM x ABL_2	BC_{2d} (n = 29)	17	13	0.55	0.46
		BC_{2e} (n = 23)	9	14	1.57	0.21
		Total (n = 52)	26	26	0.02	0.90

Mapping of the Ol-resistance in ABL_2

ABL_2 harbours resistance to *O. neolycopersici* but the origin of this resistance is unknown as it was found in an unknown germplasm of a breeding company. The inheritance of the resistance in ABL_2 was studied by applying a disease test on two BC_2 families (Table 1). All plants of the susceptible control MM showed fungal sporulation and all plants of the resistant control *L. peruvianum* LA2172 were free of fungal sporulation. The BC_2 plants could unambiguously be classified as resistant or susceptible (Table 4). In total, the two BC_2 families showed 26 resistant plants and 26 susceptible plants. The segregation of resistant to susceptible plants in each of the BC_2 families, as well as jointly, fitted a segregation ratio of 1 R : 1 S for a monogenic model (Table 4). The monogenic model was further confirmed in a BC_3S_1 population (n=80, $\chi^2 = 0.02$, P = 0.90). Thus, the complete resistance in ABL_2 is conferred by one dominant gene, designated as *Ol-6*.

BSA was performed on R and S pools of each BC_2 family to find molecular markers linked to *Ol-6*. Six AFLP markers were obtained by using 64 *Pst/Mse* AFLP primer combinations, which were present only in the resistant pool. One of the AFLP markers (M1449) was converted into a CAPS marker and mapped above RFLP marker TG178 on tomato Chromosome 6 by using a reference F_2 population of MM x *L. pennellii* LA716 (Haanstra et al. 1999b). The co-segregation between the AFLP marker M1449 and its derived CAPS marker could not be verified as no polymorphisms between MM and ABL_2 were found after screening with 24 restriction enzymes (Bai et al. 2004a). Another AFLP marker, M1346 co-segregating with *Ol-6*, was converted to a dCAPS marker (Neff et al. 1998), named dM1346. This marker was 4 cM above the CAPS marker GP79L in the *Ol-4* mapping population (Fig. 1C). For comparison of map positions between *Ol-6* and *Ol-4*, the same set of co-dominant PCR markers

used to map *Ol-4* was applied for mapping *Ol-6* in the BC_3S_1 population segregating for *Ol-6*. In this mapping population, *Ol-6* fully co-segregated with the CAPS markers GP79L and 32.5Cla that were mapped 2 cM apart from dM1346. In the *Ol-4* mapping population, CAPS markers GP79L and 32.5Cla were also fully linked to *Ol-4* (Fig. 1C). Moreover, the same marker phenotypes were observed for CAPS markers that were linked to *Ol-4* on the short arm of Chromosome 6 (digested by 24 enzymes, Bai et al. 2004a). ABL_2 showed a MM marker phenotype for CAPS markers linked to *Ol-4* on the long arm of Chromosome 6, indicating that ABL_2 likely contains a short introgressed region on the short arm of Chromosome 6. As the origin of *Ol-6* is unknown, the possibility that *Ol-6* is identical to *Ol-4* cannot be excluded. However, when NILs (near isogenic lines) containing different *Ol*-genes were compared, microscopic observation of the infection process showed clear differences between *Ol-4* and *Ol-6* (**Chapter 6**). Macroscopically, when plants were inoculated with an inoculum of 2 x 10^4 conidia.ml^{-1}, both *Ol-4* and *Ol-6* confer complete resistance (no fungal sporulation) to *O. neolycopersici*. But a lower level of resistance in the NIL of *Ol-6* than in the NIL of *Ol-4* was observed at seedling stage when plants were inoculated with an inoculum of 15 times higher concentration (3 x 10^5 conidia.ml^{-1}). These phenomena may reflect either a difference between *Ol-4* and *Ol-6*, or a difference between the genetic backgrounds of the NILs (**Chapter 6**).

DISCUSSION

In order to compare the genetic map positions of the *Ol*-genes, we have chosen an experimental approach of using common markers in different mapping populations, each of which segregated for one of the *Ol*-genes. Firstly, BSA was performed in different mapping populations to identify AFLP markers linked to the *Ol*-genes. Subsequently, the linked AFLP markers were converted to simple diagnostic PCR markers, which were used as anchors on a chromosome in a reference mapping population. Then, more RFLPs in the corresponding chromosome region were selected for conversion to simple PCR markers. Finally, common PCR markers were applied to independent mapping populations that segregated for one of the *Ol*-genes, in order to compare the genetic map positions of the *Ol*-genes. This experimental approach allowed us to generate an integrated map comprising DNA markers and different *Ol*-genes, which reveals the relative locations of five *Ol*-genes that are organized in three genetic loci on tomato Chromosome 6. We did not carry out allelism tests, since the existence of ambiguous plants during disease evaluation in allelism tests often causes difficulties for genes conferring incomplete resistance like *Ol-1* (Huang et al. 2000c). Moreover, an extremely large population would be required to distinguish two tightly linked genes, especially in a chromosome region

where recombination is reduced. This would be the case for *Ol-4* and *Ol-6* that are mapped around the *Mi* gene on Chromosome 6, where Ganal and Tanksley (1996) reported a severely suppressed recombination frequency.

All the dominant *Ol*-genes conferring resistance to *O. neolycopersici* mapped so far are exclusively located on tomato Chromosome 6 and organized in three genetic loci. Two closely linked loci are located on the long arm, one having the gene *Ol-5* and the other containing the genes *Ol-1* and *Ol-3* that have been mapped in the same chromosome region and may be allelic or tightly linked (Huang et al. 2000c). Previously, no recombinants were identified between *Ol-1* and *Ol-5* in an allelism test (Moretti and Caranta 2000), while in this study, *Ol-5* maps to a locus that is above the *Ol-1/Ol-3* locus. Very likely, *Ol-1* and *Ol-5* are tightly linked *Ol*-genes or homologues of a large gene cluster. The third locus is located on the short arm, where the genes *Ol-4* and *Ol-6* are both completely linked to the PCR marker GP79L (Fig. 2). Although *Ol-4* and *Ol-6* may be identical since the origin of *Ol-6* is unknown, they may also be allelic or linked homologues in a gene cluster, similar to the possible relationship between *Mi-1* and *Mi-9* in the same chromosomal region. *Mi-9* that confers heat-stable resistance to root-knot nematodes is closely linked to the *Mi-1* gene and is possibly a member of the *Mi-1* family (Ammiraju et al. 2003). In addition to the dominant *Ol*-genes, the region where most likely one QTL (*Ol-qtl1*) is located overlaps with *Ol-1* and *Ol-3*, as well as with *Ol-5* (Fig. 1E). The accuracy of QTL mapping did not allow a more accurate positioning of this QTL in the used mapping population (Bai et al. 2003).

The distribution pattern of the *Ol*-genes on Chromosome 6 is similar to that of *Cf* genes on Chromosome 1. On the short arm of tomato Chromosome 1, a number of *Cf* genes with different specificities have been mapped at three loci namely 'MilkyWay', 'Aurora' and 'Orion' (Haanstra et al. 1999a and 2000, Yuan et al. 2002). These *Cf*-genes belong to a large gene family of *Hcr9s* that carries multiple copies of resistance gene homologues and are located in tandem arrays (Parniske et al. 1999). For example, the 'MilkyWay' locus in both *Cf-4* and *Cf-9* lines carries five tandemly duplicated *Hcr9* genes within a 36-kb interval (Thomas et al. 1997). Molecular analysis of several other loci in tomato, such as the *Cf-2/Cf-5*, *Mi* and *I2* loci (Dixon et al. 1996, Ori et al.1997, Seah et al. 2004), also revealed the presence of tandemly arranged multigene families. Possibly, this type of organization might be common for resistance loci. It becomes an intriguing question how *Ol*-genes are organized on tomato Chromosome 6: as simple clusters with multiple genes tandemly arrayed like the *Cf* genes, or as a single locus consisting of one single gene that may carry considerable allelic variation like the *L* locus in flax. To gain more knowledge about this, the cloning of *Ol*-genes is currently underway in our laboratory. In parallel, NILs that differ only for individual *Ol*-genes in a genetic background of MM, are being tested for resistance to *O. neolycopersici* isolates from different geographic

origins. The preliminary results indicate a gene-for-gene model for the tomato-*O. neolycopersici* interaction (**Chapter 6**). In the future, our study on the characterization of the *Ol*-loci on tomato Chromosome 6 will provide more insights into the organization, specificity and evolution of the *Ol*-genes.

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Chapter 6

Different race specificities and mechanisms of resistances conferred by *Ol*-genes in tomato

Submitted.

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ABSTRACT

Tomato powdery mildew caused by *Oidium neolycopersici* has become a globally important disease of tomato. With the aim to study the host-pathogen interaction of tomato and tomato powdery mildew (*O. neolycopersici*), near isogenic lines (NILs) were generated that contain different tomato resistance genes (*Ol*-genes including *Ol-1*, *ol-2*, *Ol-3*, *Ol-4*, *Ol-5* and *Ol-6*) in the *L. esculentum* genetic background. Race specificity of resistance conferred by the *Ol*-genes was revealed by testing the NILs with local isolates of *O. neolycopersici* in different geographic locations. Moreover, the mechanism of resistance conferred by different *Ol*-genes was described microscopically. Our data suggested that the resistance to *O. neolycopersici* conferred by different *Ol*-genes can be race-specific. Microscopically, the mechanism of resistance conferred by the dominant *Ol*-genes was associated with an HR, while the mechanism of resistance governed by the recessive gene *ol-2* was associated with papillae formation.

INTRODUCTION

Oidium neolycopersici is the causal agent of the tomato powdery mildew disease. The lack of a sexual stage hampers the exact identification of this pathogen. Previously, Noordeloos and Loerakker (1989) adopted the name *O. lycopersicum* (later changed to *O. lycopersici*) for the European tomato powdery mildew fungus that appeared to come from Australia. However, the pathogen that caused the recent outbreak of tomato powdery mildew outside Australia was recently renamed to *O. neolycopersici* based on morphological features of the fungus and DNA sequences of the internal transcribed spacer (ITS) regions of the nuclear rRNA genes (Jones et al. 2001, Kiss et al. 2001). The initial events of colonization of susceptible tomato by *O. neolycopersici* have been described by Jones et al. (2000). Morphologically, *O. neolycopersici* can easily be distinguished from another species of tomato powdery mildew, *Leveillula taurica*, which occurs in subtropical regions. The mycelium of *L. taurica* grows into mesophyll of the leaf and is visible on the down side of the leaf, while *O. neolycopersici* mainly grows on the upper side and usually does not penetrate into the mesophyll (Lindhout et al. 1994a).

The cultivated tomato is susceptible, but various levels of resistance to *O. neolycopersici* have been observed in different wild *Lycopersicon* species (Lindhout 1994a, Mieslerová et al. 2000). Macroscopically, resistance to *O. neolycopersici* in wild tomato species is characterized by strongly restricted mycelial growth and lack of sporulation (Lindhout 1994a). Microscopically, the hypersensitive reaction (HR) is the major mechanism of resistance to *O. neolycopersici* in *Lycopersicon* species (Huang et al. 1998, Mieslerová et al. 2004). It has been suggested that the level of resistance to *O. neolycopersici* may be affected by the genetic background (Huang et al. 1998, Mieslerová and Lebeda 1999). Little is known about the genetic variation within *O. neolycopersici*. A set of *Lycopersicon* spp. genotypes showed differential reactions with isolates originating from Czech Republic, Germany, the Netherlands and Great Britain (Lebeda and Mieslerova 2000). Recently, Kashimoto et al. (2003) reported that one resistant tomato cultivar bred in the Netherlands is susceptible to the Japanese *O. neolycopersici* isolate KTP-01. These results indicate that the resistance to *O. neolycopersici* is race-specific. However, as long as a differential series of tomato genotypes with well-defined resistances is lacking, it will remain hard to conclude that different races of *O. neolycopersici* exist.

Our research aim is to study the interaction between resistance genes in tomato and different *O. neolycopersici* isolates. We have mapped five qualitative resistance genes (*Ol*-genes) introgressed from at least two *Lycopersicon* species (**Chapter 5**), and three QTLs (*Ol-qtls*) that originated from *L. parviflorum* G1.1601 (Bai et al. 2003). Remarkably, all the dominant *Ol*-genes mapped so far are located in three genetic loci on tomato Chromosome 6. On the long arm, one locus contains the genes *Ol-1* and *Ol-3*, originating from *L. hirsutum*

G1.1560 and G1.1290, respectively (Lindhout et al. 1994b, Huang et al. 2000c). Another locus on the long arm contains *Ol-5*, a gene originating from *L. hirsutum* PI247087 (Latterot et al. 1995). On the short arm of Chromosome 6, one locus contains two genes *Ol-6* (unknown origin) and *Ol-4* (originating from *L. peruvianum* LA2172, Bai et al. accepted). In addition, a recessive resistance gene (*ol-2*) that originates from *L. esculentum* var *cerasiforme* has been mapped on tomato Chromosome 4 (Ciccarese et al. 1998, De Giovanni et al. 2004). To study the interaction between the *Ol*-genes in tomato and different *O. neolycopersici* isolates, near isogenic lines (NILs) were generated by repeated backcrossing. Each NIL contains an unique *Ol*-gene in a genetic background of the susceptible *L. esculentum* cv. Moneymaker. These NILs were tested with local isolates of *O. neolycopersici* in different locations in the world. Moreover, by using these NILs, the resistance mechanisms associated with the *Ol*-genes were studied microscopically. In the present paper, we report different race specificities and mechanisms of resistances conferred by the *Ol*-genes (*Ol-1, ol-2, Ol-3, Ol-4, Ol-5* and *Ol-6*).

MATERIALS AND METHODS

Plant materials

Advanced breeding lines (ABLs) that contain *Ol-1*, *Ol-3* and *Ol-6* genes in a genetic background of *L. esculentum* have been provided by different breeding companies, and an ABL with *Ol-5* has been obtained from INRA (Montfavet, France). These ABLs and *L. peruvianum* LA2172 (harboring *Ol-4*) have been crossed to susceptible *L. esculentum* cv. Moneymaker (MM). By using MM as a recurrent parent, backcross generations have been produced. Resistant plants were selected by performing disease tests on each backcross generation (BC_1, BC_2 and BC_3). For each dominant *Ol*-gene (*Ol-1*, *Ol-3*, *Ol-4*, *Ol-5* and *Ol-6*), 96 BC_3S_1 plants from different BC_3 families were subjected to marker assisted selection (MAS) to select homozygous resistant and susceptible plants by using simple PCR markers linked to the *Ol*-genes (**Chapter 5**). The selected BC_3S_1 plants were screened with 12 AFLP primer combinations to characterize the genetic background. The resistant BC_3S_1 plants were further selfed to produce BC_3S_2 lines that were considered as *Ol*-NILs in this study. In addition to the *Ol*-NILs of the dominant *Ol*-genes, an F_3 line of *L. esculentum* cv. Marmande x *L. esculentum* var. *cerasiforme* carrying the recessive *ol-2* gene was kindly provided by Dr. Luigi Ricciardi (University of Bari, Italy) and further designed 'NIL-*ol-2'*.

Fungal material

O. neolycopersici isolates from the Netherlands (*On*-Ne), Hungary (*On*-Hu), the Czech Republic (*On*-Cz), Florida USA (*On*-Fl), France (*On*-Fr) and Italy (*On*-It) were collected locally from infected tomato plants and used in this study (Table 1).

Table 1. Local isolates of *O. neolycopersici* and places for the disease tests

Name of isolates	Location	Country	Institution	Local isolate name
On-Ne	Wageningen	the Netherlands	Wageningen University	-
On-Hu	Budapest	Hungary	Plant Protection Institute of the Hungarian Academy of Sciences	BP-P5
On-Cz	Olomouc	Czech Republic	Palacky University	Isolate 1/98
On-It	Bologna	Italy	Nunhems Seeds	- (obtained from Prof. Mario Marte, University of Perugia)
On-Fr (A)	Avignon	France	Rijk Zwaan	76 local
On-Fr (B)	Avignon	France	De Ruiter Seeds	-
On-Fl	Florida	USA	University of Florida	-

Disease test

The experimental set-up for all disease tests on the NILs was a randomized block design with two blocks. Each block contained five plants of all six *Ol*-NILs and MM. The inoculation was performed as described by Bai et al. (2003), and inoculated plants were grown in a greenhouse at 20±5°C with 30-90% RH. The inoculated plants were scored according to the following disease index (DI), 0 = no visible sporulation, 1 = very few fungal spots surrounded by necrosis (weak sporulation), 2 = moderate number of fungal spot (moderate sporulation), 3 = very high number of fungal spots (heavy sporulation). Four evaluations on fungal development were performed in time with an interval of two days. The first one was on the day when inoculated MM plants started showing symptoms. The DI of each line was calculated by averaging the disease index of all tested plants over the last three time evaluations.

For the histological study, a disease test was carried out with the isolate *On*-Ne in a climate chamber at 20±1°C with 70±10% RH and a 16 hour photoperiod. A pilot experiment was carried out on MM plants infected with *O. neolycopersici* isolate (*On*-Ne) by using print- or

spray- inoculation methods. The print-inoculation was done by direct contact (gently pressing) of heavily sporulating MM leaves to leaves of plants to be inoculated. The spray-inoculation was performed by spraying plants with a spore suspension of $3x10^5$ conidia.ml^{-1}. In order to investigate the resistance mechanism, another experiment was performed with six *Ol*-NILs and MM. Eight plants per line were randomly arranged and were inoculated by the spray-inoculation method (six plants for microscopic observation and two plants for macroscopic scoring). The 3rd true leaf (fully expanded) of one-month-old plants was inoculated.

Sampling and staining

In the pilot experiment, leaf segments (two per plant) of 1x3 cm^2 were sampled from one of the print-inoculated MM plants at 41, 65 and 89 hours post inoculation (hpi). In the experiment with NILs, two plants per line and per time point (41, 65 and 89 hpi) were used for sampling leaf segments (three leaf segments per plant). The sampled leaf segments were stained with chloral hydrate/trypan blue as described by Huang et al. (1998).

Micro- and macroscopic evaluation

A conidiospore was defined as germinated when it produced either a germ tube of at least half the length of the spore or a germ tube with a primary appressorium. An infection unit (IU) was defined as a germinated spore that produced at least a primary appressorium. Twenty IU per leaf segment were scored. Number of hyphae, appressoria and haustoria per IU were recorded, as well as the presence of cell necrosis and papillae formation. DI was recorded at 11 and 14 days post inoculation (dpi).

Molecular marker analysis

Total DNA was extracted from leaves of the plants prior to inoculation by using a rapid CTAB DNA isolation method as described by Brugmans et al. (2003) and the AFLP fingerprints were generated on a LI-COR 4200 DNA sequencer as described by Bai et al. (2003). The detailed information for the simple PCR markers used in MAS has been presented in **Chapter 5.**

RESULTS

Development of near isogenic lines

To develop NILs that only differ for *Ol*-genes, resistant donor accessions were crossed to the susceptible MM. Homozygous BC_3S_1 plants (resistant and susceptible) of these crosses were selected (see Materials and Methods) and subjected to AFLP analysis in order to compare their

genetic background with the recurrent parent MM. Most BC_3S_1 plants were genetically more than 90% similar to the recurrent parent. For example, with 12 AFLP primer combinations, 30 AFLP markers were obtained that were specific for *L. hirsutum* G1.1560, the donor of *Ol-1*. Of the 30 AFLP marker alleles from the donor parent, only three were present in the BC_3S_1 plants containing *Ol-1*. Two fully co-segregated with the resistance indicating that these two markers were located on the *Ol-1* introgression, while the third marker did not co-segregate with *Ol-1*. Similar results were obtained for the BC_3S_1 plants containing *Ol-3, Ol-5,* and *Ol-6* genes (data not shown). But for the BC_3S_1 plants containing the *Ol-4* gene, the result was different. In these plants, 48 AFLP marker alleles in total were from the donor parent and 11 of these wild donor alleles (11 of 48) still segregated in the BC_3S_1 plants. This result can be explained by the fact that these BC_3S_1 plants were derived from an interspecific-cross between MM and *L. peruvianum*, while the other crosses were intraspecific between MM and ABLs that were already genetically similar to MM. Thus, further backcrosses to MM are needed to get a more isogenic NIL-*Ol-4*.

BC_3S_2 lines from selfed homozygous resistant BC_3S_1 plants are referred to as *Ol*-NILs and further characterized in this study, as well as the line NIL-*ol-2* (see Materials and Methods).

Race specificities of resistances conferred by the Ol-genes

Initially, to investigate the interactions between the NILs and the *O. neolycopersici* isolates we considered to test the NILs in one location with isolates collected from different parts of the world. However, in past research we experienced cross contamination of different powdery mildews during maintenance and propagation even though spore-proof growth cabinets were used (PL, unpublished). Finally, we decided to test the NILs with local isolates in different geographic locations in order to overcome the problem of cross contamination of *O. neolycopersici* (Table 1). As the tests were carried out in different places, variance could be introduced due to different test conditions and human interpretations on the level of resistance. To reduce this variance, test conditions were standardized and detailed descriptions with symptom pictures for disease evaluation were provided to all test sites. Before distributing the NILs, a disease test with isolate *On*-Ne was carried out on these NILs (20 plants per line and MM as a susceptible control) to confirm that the NILs were homogeneously resistant. At each location, the variation in disease index (DI) among the tested plants within each line was small (data not shown), and a mean DI was calculated for each line by averaging the DI of all tested plants (see Materials and Methods). Lines were considered as resistant when DI≤2 (completely resistant if DI ≤1 and incompletely resistant when 1<DI≤2) and as susceptible when DI >2 (Table 2).

Table 2. Interactions of *Ol*-NILs with different isolates of *O. neolycopersici*

***Ol*-genes**				***Oidium neolycopersici* isolates**						
Genotype	**Origin**	**Locus**	**Chromosome**	***On*-Fl**	***On*-Hu**	***On*-Fr (A)**	***On*-Fr (B)**	***On*-Cz**	***On*-Ne**	***On*-It**
NIL- *Ol-1*	*L. hirsutum*	*Ol-1/Ol-3*	6, long arm	R*	IR	R	R	R	R	R
NIL- *Ol-3*	*L.hirsutum*	*Ol-1/Ol-3*	6, long arm	R	R	R	R	R	R	R
NIL- *Ol-5*	*L. hirsutum*	*Ol-5*	6, long arm	R	IR	R	R	R	R	R
NIL- *Ol-4*	*L. peruvianum*	*Ol-4/Ol-6*	6, short arm	R	R	R	R	S	R	R
NIL- *Ol-6*	Unknown	*Ol-4/Ol-6*	6, short arm	R	R	R	R	S	R	R
NIL- *ol-2*	*L. esculentum*	*ol-2*	4	R	R	R	R	R	R	R/IR
Moneymaker	*L. esculentum*			S	S	S	S	S	S	S

*R: completely resistant (DI ≤ 1); IR: incompletely resistant (1 < DI ≤ 2); S: susceptible (DI >2).

Table 3. Development of *O. neolycopersici* on *Ol*-NILs[a]

Genotype	**1st haus. per IU[b] (%)**	**necrotic cell per 1st haus. (%)**	**necrotic cell per 2nd haus. (%)**	**1st papilla per 1st appre. (%)**	**2nd papilla per 2nd appre. (%)**	**1st papilla per 1st haus. (%)**	**2nd papilla per 2nd haus. (%)**	**No. of hyphae per IU**	**No. of 2nd appre. per IU**	**No. of 2nd haus. per IU**
Money-maker	93 a	0 a	2 a	0	0	0	0	4.9 a	4.9 a	2.5 a
NIL-*Ol-1*	86 a	32 b	25 b	0	0	0	0	3.8 b	4.6 a	2.2 ab
NIL-*Ol-3*	84 a	33 b	27 b	0	0	0	0	3.4 b	4.4 a	2.5 a
NIL-*Ol-5*	91 a	31 b	24 b	0	0	0	0	4.0 b	4.1 a	1.8 b
NIL-*Ol-6*	93 a	81 c	54 c	0	0	0	0	2.3 c	3.5 a	1.7 b
NIL-*Ol-4*	56 b	100 d	100 d	0	0	0	0	0.3 e	0.3 b	0.2 c
NIL-*ol-2*	42 b	0 a	0 a	67	49	90	94	1.4 d	1.5 b	0.3 c

[a] Means followed by a different letter are significantly different at the 5% level (P<0.05), determined by Genstat program. Data transformation (*arcsine*) has been done for percentages.

[b] IU, infection unit; 1st, primary; appre., appressorium; haus., haustorium; 2nd, secondary.

NIL-*Ol-1* and -*Ol-5* were incompletely resistant to the Hungarian isolate *On*-Hu and were completely resistant to the rest of the isolates tested. NIL- *ol-2* and -*Ol-3* were resistant to all isolates, however in Italy NIL-*ol-2* was completely resistant to *On*-It in one test but incompletely resistant in another test. The difference between the two tests may be caused by differences in testing conditions, spore quality of the inoculum or subjective evaluations by different persons. NIL-*Ol-4* and -*Ol-6* showed complete resistance to all the isolates, except for the Czech isolate *On*-Cz. The susceptibility of NIL-*Ol-4* and -*Ol-6* to *On*-Cz was confirmed by a repeated disease test. In conclusion, our data suggested the existence of different race specificities of resistances conferred by the *Ol*-genes.

Different resistance mechanisms associated with the Ol-genes

To obtain good preparations for studying resistance mechanisms, a pilot experiment was performed with two inoculation methods. The print-inoculation resulted in a high spore density with a very heterogeneous spore distribution. Many spores clustered and branching hyphae intertwined, which made microscopic observations difficult and unreliable. The spray-inoculation gave an equal and dense spore distribution, enabling sufficient numbers of infection units (IU) to be evaluated. In the susceptible MM, IU was well established at 65 hpi showing branching hyphae, primary and secondary haustoria. Thus, for the experiment with NILs, spray-inoculation method was used and microscopic observations were done on samples collected at 65 hpi.

To verify whether the resistance conferred by these *Ol*-genes remained effective under a high spore concentration (3×10^5 conidia/ml, 15 times higher than normal), sporulation was recorded on two plants per line. Susceptible control MM plants sporulated heavily (DI = 3), while the NILs containing the genes of *Ol-1*, *Ol-3*, *Ol-4* and *Ol-5* showed similar resistance level as in disease tests with a normal inoculum density (2×10^4 conidia.ml^{-1}). The NILs of *ol-2* and *Ol-6* showed some fungal spots surrounded by necrosis (DI = 1), compared to no-symptoms (DI = 0) upon a spore density of 2×10^4 conidia.ml^{-1}. Therefore, we concluded that all the NILs showed a similar resistance level as under normal inoculum dose.

The fungal development was microscopically evaluated on the NILs and MM (Table 3). On all lines, all germ tubes had formed primary appressoria at 65 hpi. Compared to MM, NILs containing *Ol-1*, *Ol-3*, *Ol-5* and *Ol-6* showed similar frequencies of both appressorium and haustorium formation, but significantly lower numbers of hyphae per IU. In MM, no epidermal cells with a primary haustorium (Fig. 1A) became necrotic, and only 2% of the cells with secondary haustoria showed necrosis that is typical for the hypersensitive reaction (HR). In contrast, the HR frequency for the primary and the secondary haustoria was about 30% in NIL-*Ol-1*, -*Ol-3* and -*Ol–5*. In NIL-*Ol-6*, about 80% of the cells with primary haustoria and 50% of

the cells with secondary haustoria showed HR, both significantly higher than NIL-*Ol-1*, -*Ol-3* and -*Ol–5*. In NIL-*Ol-1* and -*Ol-3*, two types of necrotic cells were observed. The first type (Type-1) showed a fully shriveled epidermal cell with a shriveled haustorium (Fig. 1B). The second type (Type-2) showed an epidermal cell with many particles and a normal or shriveled haustorium (Fig. 1C). In NILs of *Ol-5* and *Ol-6*, Type-2 was predominant. NIL-*Ol-4* showed a significant reduction in the frequency of haustorium formation and a very high frequency of HR (type-1, Fig. 1B) that was associated with an almost complete arrest of further fungal development (Table 3).

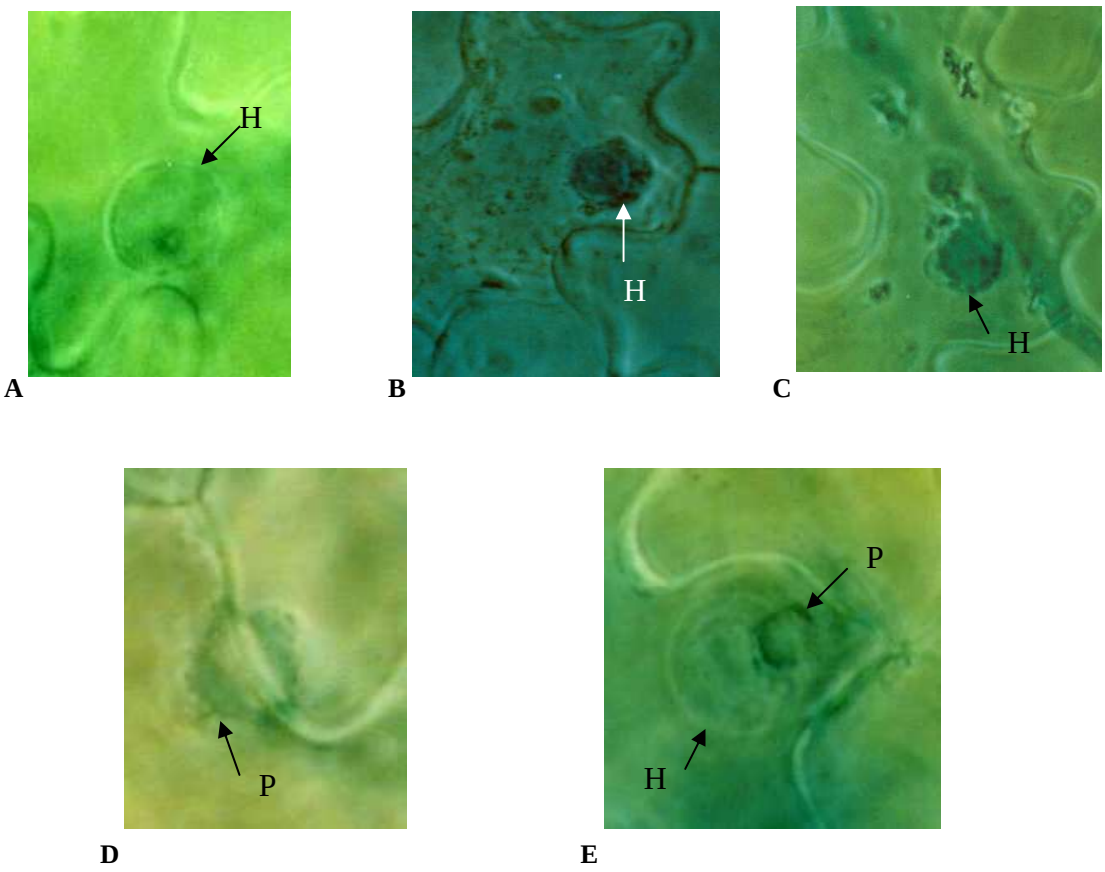

Fig. 1. Phenotypes of haustoria, necrotic cells and papillae**. A,** a normal haustorium (indicated as H) in an epidermal cell. **B,** an abnormal haustorium in a shriveled epidermal cell (type-1 necrosis). **C,** an abnormal haustorium in an epidermal cell with many particles (type-2 necrosis). **D,** Papillae formation (indicated as P) beneath the appressorium. **E,** Papillae formation beneath the haustorium.

Remarkably, only in NIL-*ol-2*, papillae formation was observed beneath the appressoria, while HR was completely absent (Table 3, Fig. 1D). In this line, a lower number of infection units (about 10 IU/cm^2) was observed than in the other NILs (about 30 IU/cm^2), which suggests inhibited spore germination. Further fungal growth was significantly reduced (Table 3). More than 90% of the haustoria were associated with papillae formation around the haustorial neck (Fig. 1E). From this experiment, it is not clear whether the papillae penetrated by the fungus to produce a haustorium, or the material had been deposited around the haustorial neck after the haustorium formation. Also it is not possible to determine whether the papilla associated haustorium formation failure was due to these papillae (Fig. 2D), or the papillae were formed after the haustorium formation had been aborted due to the other mechanism.

DISCUSSION

In the present study, our data indicate that the resistance conferred by some *Ol*-genes is race-specific, and suggest the existence of different *O. neolycopersici* races. Previously, Kashimoto et al. (2003) described that a resistant tomato cultivar "Grace" bred in the Netherlands was susceptible to the Japanese isolate KTP-01. Similarly, Lebeda and Mieslerova (2000) reported that the British isolate of *O. neolycopersici* had specific and higher pathogenicity to certain *Lycopersicon* accessions than other isolates. However, this is the first study on the interaction of tomato and *O. neolycopersici*, using well defined plant material containing different *Ol*-genes in a similar genetic background and with a range of *O. neolycopersici* isolates. To further investigate the race specificity of the resistance conferred by the *Ol*-genes, these NILs will be tested in more locations including Japan and Great Britain.

The resistance to *O. neolycopersici* conferred by the dominant *Ol*-genes was posthaustorial and associated with HR. In this study, HR was restricted to single cells, except for NIL-*Ol-4* where necrosis was also observed in some cells adjacent to haustorium-invaded cells (spreading necrosis). Previously, spreading necrosis was reported in *L. hirsutum* G1. 1290 (the donor of *Ol-3*, Huang et al. 1998), and other *Lycopersicon* species (Mieslevorá et al. 2004). These different observations may be caused by different genetic backgrounds of the plant materials used, as the material used in the previous studies were less isogenic for *L. esculentum* background than in the present study. In NIL-*Ol-4*, HR was complete at the stage of primary haustoria and hardly any hyphae or secondary appressoria were formed (even at 89 hpi), suggesting that HR was very effective to arrest early fungal development in this line. In the rest of the NILs, not all epidermal cells with haustoria became necrotic and fungal development was generally not stopped but strongly retarded, as reported by Huang et al. (1998) and Mieslevorá et al.(2004). Race-specific resistance is often associated with HR (Dangle and Jones, 2001;

Lindhout, 2002), which is now also proven for the dominant monogenic resistance genes *Ol-4* and *Ol-6*. As resistance conferred by *Ol-1*, *Ol-3* and *Ol-5* is associated with HR, it is not unlikely that the resistance will be overcome by certain races of *O. neolycopersici*.

In this study, it was revealed that the recessive *ol-2* gene is associated with papillae formation and not with HR. In NIL-*ol-2*, the haustorium formation and the fungal development were significantly reduced although the fungal penetration was not fully prevented. There are a few other examples of monogenic resistances that are based on non-HR defense mechanism. The recessive *mlo* gene conferring resistance to *Blumeria graminis* f. sp. *hordei* in barley is involved in papillae formation that prevents fungal haustorium formation and infection (Lyngkjaer et al. 2000), while the dominant *Lr*34 gene conferring incomplete resistance to *Puccinia triticina* in wheat is also associated with reduced haustorium formation and not with HR (Rubiales and Niks, 1995). The resistances governed by both *mlo* and *Lr34* are race-non-specific. The resistance conferred by the recessive gene *ol-2* is non-HR and is not race-specific concerning the *O. neolycopersici* isolates tested so far. Possibly, like *mlo* gene, *ol-2* governs race-non-specific resistance to *O. neolycopersici* and as this mechanism is not based on HR it might be more durable than the dominant *Ol*-genes (Lindhout 2002).

The closely linked *Ol*-genes on tomato Chromosome 6 had a similar race specificity and HR reaction. On the long arm of Chromosome 6, closely linked *Ol*-genes (*Ol-1*, *Ol-3* and *Ol-5*) that originate from *L. hirsutum* were effective to all isolates tested and had similar HR reaction against fungal development. On the short arm of Chromosome 6, the genes *Ol-4* and *Ol-6* were mapped at the same locus and showed identical specificity to the tested *O. neolycopersici* isolates. The difference in the efficiency in HR between NIL-*Ol-4* and NIL-*Ol-6* (Table 3) and the different types of hypersensitivity (Fig. 1) may reflect either a difference between the two genes (*Ol-4* and *Ol-6*), or a difference in the genetic backgrounds of the NILs. NIL-*Ol-4* contains a larger introgressed chromosome segment from *L. peruvianum* (covering both the long arm and the short arm of Chromosome 6), than the NIL-*Ol-6* in which the donor chromosome segment is very small and confined to the short arm. Resistance genes in plants are often represented by linked clusters of resistance genes or analogues or by many alleles of a single locus. Frequently, members of the same complex gene cluster confer resistance to different pathotypes of the same pathogen species (Hulbert et al. 2001, Michelmore and Meyers 1998). For example, the *Mla* gene complex in barley, the *Cf* gene clusters in tomato and the *Dm3* cluster in lettuce (Jϕrgensen 1994, Meyers et al. 1998, Parniske et al. 1999). Now, it becomes an intriguing question how *Ol*-genes are organized on tomato Chromosome 6 and how they have originated. Cloning of these *Ol*-genes is in progress, in order to provide insight into the structure of the *Ol*-genes. In parallel, genes involved in defense response are being revealed by gene expression profiling of *Ol*-NILs infected with *O. neolycopersici*.

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Chapter 7

General discussion

Hot spots of resistance

Hot spots of resistance are defined as chromosomal regions, which harbor genes that confer resistance to several unrelated pathogens. The most striking example is the hot spot in potato that contains the nematode resistance gene *Gpa2* and the virus resistance gene *Rx1* at the same haplotype in a chromosomal region of 115 kb (Van der Vossen et al., 2000). In this thesis (**Chapter 5**), *Ol-4* and *Ol-6* are mapped in a hot spot on the short arm of tomato Chromosome 6. This region contains two *Cf* genes (*Cf-2* and *Cf-5*) conferring resistance to *Cladosporium fulvum*, the *Ty-1* gene conferring resistance to tomato yellow leaf curl virus and the *Mi* gene governing resistance to three very different organisms: root-knot nematodes, aphids and whitefly (Chagué et al. 1997, Dickinson et al. 1993, Nombela et al. 2003). Interestingly, the NILs of *Ol-4* and *Ol-6*, as well as *L. peruvianum* LA2172 (donor of *Ol-4*), are resistant to both *O. neolycopersici* and the root-knot nematode *Meloidogyne incognita*, while the breeding line "Motelle", donor of the *Mi* gene, is susceptible to *O. neolycopersici*. This result may suggest that either *Ol-4/Ol-6* has a pleiotropic effect, or *Mi* and *Ol-4*/*Ol-6* are very closely linked and present at the same haplotype on the short arm of tomato Chromosome 6 (Bai et al. unpublished). Genes for resistance (R genes) to diverse pathogens cloned so far share several features and most R genes encode a leucine-rich repeat (LRR) region (Michelmore and Meyers 1998). It has been illustrated that relatively small changes in R gene sequence can result in resistance against entirely different pathogen species (Wang et al. 1998, Ellis et al. 1999). For example, the proteins encoded by the above mentioned *Gpa2* and the *Rx1* genes share an overall homology of over 88% (amino-acid identity) and belong to one class of resistance genes that contains a leucine-zipper, nucleotide-binding site and leucine-rich repeat (LZ-NBS-LRR, Van der Vossen et al. 2000), while one sequence of the *Mi* gene confers resistance to three different organisms. On the other hand, genetic linkage of R genes does not necessarily imply sequence similarity. Within the hot spot where *Ol-4/Ol-6* maps, *Mi* and *Cf* genes have been cloned. *Mi* encodes a protein that belongs to a class of LZ-NBS-LRR (Milligan et al. 1998), while *Cf-2* and *Cf-5* genes encode proteins with a common feature of LRR and transmembrane (TM) domains (Dixon et al. 1996, 1998). Cloning of *Ol-4/Ol-6* is currently being carried out and sequence analysis of these R genes will increase our understanding of their genetic relationship and possible evolution.

Organization and evolution of disease resistance genes

In plants, resistance (R) genes occur in a number of different genomic organizations (Michelmore and Meyers 1998, Richter and Ronald 2000). The simplest arrangement is a locus consisting of a single R gene, which may show considerable allelic genetic variation, like the *L* locus of flax, consisting of a single gene with 13 distinct alleles. More frequently, R genes in plants are members of multigene families and located in tandem arrays, forming clusters like the *Mi*, *I-2* and *Cf* gene clusters in tomato, the *Mla* cluster in barley and the *Xa21* gene cluster in rice. All the dominant *Ol*-genes conferring resistance to *O. neolycopersici* mapped so far are exclusively located on tomato Chromosome 6 and organized at three genetic loci. Two closely linked loci are located on the long arm (containing *Ol-1/ Ol-3* and *Ol-5*, respectively) and the third locus is located on the short arm (consisting of *Ol-4* and *Ol-6*) (**Chapter 5**). The question is now arising: are *Ol*-genes on tomato Chromosome 6 organized as simple clusters with multiple genes arranged in tandem, or as a single locus consisting of one single gene that may carry considerable allelic variation?

Interestingly, *Ol*-genes that are genetically linked on tomato Chromosome 6 have the similar specificity to the isolates of *O. neolycopersici* tested in our study (**Chapter 6**). For example, *Ol*-genes (*Ol-4* and *Ol-6*) mapped on the short arm have the same race specificity, while no race specificity has been detected for *Ol*-genes (*Ol-1*, *Ol-3* and *Ol-5*) linked on the long arm. For many R genes, sequence exchange and diversifying selection are likely the two principle mechanisms that create R genes with new specificities (Michelmore and Meyers, 1998, Richter and Ronald, 2000). However, the existence of *RPM1* and *Pto* genes in different species of *Arabidopsis* and *Lycopersicon*, respectively, suggests that R genes with particular specificities already existed before the species diverged (Stahl et al. 1999, Vleeshouwers et al. 2001). In most plant-pathogen systems, a resistance gene introgressed from wild germplasm initially confers resistance to all the genotypes of a pathogen, but becomes ineffective later after large scale growing of cultivars containing the resistance gene. However, this is not typical for the resistance to *O. neolycopersici* in tomato. All the commercial tomato cultivars released before 1990s are susceptible to *O. neolycopersici* (Fletcher et al. 1998, Lindhout et al. 1994a&b). Since resistant cultivars were not widely used, selection pressure on the pathogen has virtually been absent. The fact that race-specific resistance to *O. neolycopersici* was identified in wild *Lycopersicon* species may suggest that the appearance of the *Ol*-genes with particular specificities already existed before the species diverged. In order to reveal the organization and evolution of the *Ol*-genes on Chromosome 6, cloning of the *Ol*-genes and sequence analysis are necessary.

Race-specific resistance and relations with different resistance mechanisms

Qualitative resistances are often associated with HR and are race-specific (Dangle and Jones 2001, Lindhout, 2002), which is also true for some dominant resistance genes (*Ol*-genes) to *O. neolycopersici* (**Chapter 6**). Few examples have been reported on monogenic resistances that are based on papillae formation and represent a non-HR defense mechanism, like the recessive *mlo* gene in barley (Lyngkjaer et al. 2000) and the dominant *Lr*34 gene in wheat (Rubiales and Niks 1995). The resistances governed by both *mlo* and *Lr34* are race-non-specific. Papillae are defined as cell wall appositions at the site of pathogen penetration, which are associated with failed fungal haustorium formation and infection. Such a prehaustorial resistance that is associated with papillae formation is prominent in both quantitative race-non-specific resistance and in non-host resistance (Niks and Rubiales 2002). These resistance types appear to be durably effective. In tomato, the resistance conferred by the monogenic recessive gene *ol-2* was associated with papillae formation and was not associated with HR. So far, NIL-*ol-2* is resistant to all the *O. neolycopersici* isolates tested, although resistance to the Italian isolate (*On*-It) may not always be 100% effective (**Chapter 6**). Possibly, *ol-2* governs race-non-specific resistance to *O. neolycopersici*.

Quantitative resistance is presumed to act in a race non-specific manner and can be due to a wide variety of mechanisms (Lindhout 2002). Many reports describe co-localization of QTLs with loci that are involved in HR resistance (**Chapter 2**). This suggests that QTLs may be involved in HR or any other resistance mechanism. In the barley-*Puccinia hordei* plant-pathosystem, quantitative resistance is not associated with HR, but with frequent failure of haustorium formation (Niks and Rubiales 2002). The three *Ol-qtls* identified from *L. parviflorum* G1.1601 together confer a high level of resistance to *O. neolycopersici* (**Chapter 2**). The map positions of two QTLs (*Ol-qtl1* and *Ol-qtl2*) co-localized with monogenic resistance loci for tomato powdery mildew, while *Ol-qtl3* does not co-localize with a locus for resistance to tomato powdery mildew as far as we know. Microscopically, the resistance in *L. parviflorum* G1.1601 was less clearly associated with HR, suggesting that a different resistance mechanism may play a role (Huang et al. 1998). Thus, these *Ol*-qtls would be good candidates to study resistance mechanisms for quantitative resistance.

Perspectives for breeding tomato cultivars resistant to *O. neolycopersici*

Resistance to *O. neolycopersici* is an important trait in tomato breeding. In the present research, the genetic basis for several resistances to *O. neolycopersici* originating from wild *Lycopersicon* species was revealed, and the corresponding resistance genes were mapped on the tomato genome. As the resistance conferred by *Ol*-genes can be race-specific (**Chapter 6**), the NILs of the *Ol*-genes and *Ol*-qtls represent very useful genetic stocks to be exploited in breeding

programs. The simple PCR markers generated in this study are useful diagnostic markers in practical breeding programs for marker assisted selection.

Future research

The research described in this thesis is being continued. Currently, we are testing the NILs of monogenic *Ol*-genes with local isolates of *O. neolycopersici* in more locations (**Chapter 6**). Meanwhile, generation of NILs for individual *Ol*-qtl or combinations of different *Ol*-qtls is ongoing. These NILs will be tested with different races of *O. neolycopersici* to study their race-specificity. Also, the resistance mechanisms conferred by *Ol*-qtls will be characterized by histological analysis. Further, profiling of differentially expressed genes associated with resistance governed by the *Ol*-genes and *Ol*-qtls is revealed by cDNA-AFLP analysis. In parallel, fine mapping of the *Ol*-genes and *Ol*-qtls is being performed aiming at cloning these genes. At the moment, sequencing of a part of tomato Chromosome 6 has started, which will facilitate cloning of the *Ol*-genes and *Ol-qtl1* on this chromosome.

In conclusion, the research in this thesis provides a solid basis for studying plant-pathogen interaction of tomato-tomato powdery mildew in the future and facilitates the breeding of cultivars resistant to *O. neolycopersici*. Furthermore, it will stimulate scientific research on the function and structures of the *Ol*-genes and *Ol*-qtls, which may allow understanding of the relationship between genes underlying qualitative and quantitative resistances.

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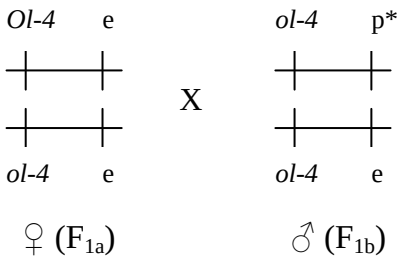
Appendix

Estimation of recombination frequency between *Ol-4* and (multi-allelic) markers from the pseudo-F_2 population

Altogether we used four types of markers (all possible marker-allele configurations in the F_2 population), for each of which we discuss the linkage analysis below.

(A) Markers that distinguish between the alternative *L. peruvianum* alleles (p, p*), but only one of these alleles is distinct from the *L. esculentum* allele (p* ≠ p = e). Marker By-4*/Hyp*CH4IV is of this type (Table 4 and Fig. 2 in **Chapter 4**). The configuration of the cross between the two F_1 genotypes (F_{1a} and $F_{1b,}$ see Fig. 1 in **Chapter 4**) looks as follows:



Since neither of the parents is heterozygous at both loci, markers of this type are not informative for linkage. In fact the loci behave as if they segregate independently (see below). Not being aware of this non-informative nature, the joint segregation can be misinterpreted as 'unlinked loci'.

Later on we will have to introduce a parameter for preferential transmission of marker alleles. Let this parameter be β for allele transmission rate in the male parent ($\beta = 0.5$ corresponds to a 1 : 1 Mendelian ratio). This leads to the following table of gamete combinations and their frequencies:

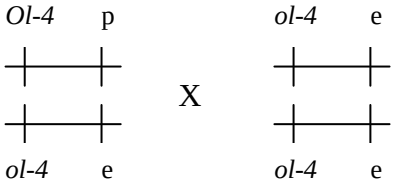
♀ \ ♂	β *ol-4 p**	$1-\beta$ *ol-4 e*
$\frac{1}{2}$ *Ol-4 e*	$\frac{1}{2}\beta$	$\frac{1}{2}(1-\beta)$
$\frac{1}{2}$ *ol-4 e*	$\frac{1}{2}\beta$	$\frac{1}{2}(1-\beta)$

The following two-way table shows genotypes for the progeny (numbers in parentheses are the observed numbers for the marker By-4/*Hyp*CH4IV from Table 4 in **Chapter 4**).

	Ol-4/ol-4	*ol-4/ol-4*	Sum
*e/p**	$\frac{1}{2}\beta$ (78)	$\frac{1}{2}\beta$ (75)	β (153)
e/e	$\frac{1}{2}(1-\beta)$ (15)	$\frac{1}{2}(1-\beta)$ (18)	$1-\beta$ (33)
Sum	$\frac{1}{2}$ (93)	$\frac{1}{2}$ (93)	1 (186)

Despite the distorted segregation, we indeed observe independent segregation between this marker and the *Ol-4* locus. The distortion parameter β is estimated as $\hat{\beta} = \frac{153}{186} = 0.8226$.

(B) Markers that distinguish between the two alternative *L. peruvianum* alleles (p, p*), but only one of these is distinct from the *L. esculentum* allele (p ≠ p* = e). (This is the 'mirror' situation of (p* ≠ p = e), discussed above.) Marker By-4/*Apo*I is of this type. The configuration of the cross looks like:



♀ (F_{1a}) ♂ (F_{1b})

This represents the classical test cross configuration, from which recombination frequency (*r*), is readily estimated by counting the recombinant genotypes among the offspring. However, for the sake of completeness we introduce a parameter, α, for preferential transmission of marker alleles by the female parent (α = 0.5 corresponds to a 1 : 1 ratio). This leads to the two-way table of genotype frequencies below (observed numbers are in parentheses, which are taken from Table 4 (**Chapter 4**) for marker By-4/*Apo*I).

	Ol-4/ol-4	*ol-4/ol-4*	Sum
e/p	$\alpha(1-r)$ (85)	$\boxed{\alpha r}$ (4)	α (89)
e/e	$\boxed{(1-\alpha)r}$ (8)	$(1-\alpha)(1-r)$ (89)	$1-\alpha$ (97)
Sum	(93)	(93)	1 (186)

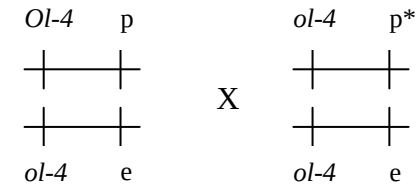
Notice that the boxed genotypes together occur with frequency *r*. So we estimate

$\hat{r} = \frac{12}{186} = 0.0645$, and

$\hat{\alpha} = \frac{89}{186} = 0.4785$.

We note that α is close to 0.5, indicating that no significant preferential transmission of alleles by the female parent occurs at this marker locus.

(C) Markers that distinguish between the two *L. peruvianum* alleles, as well as the *L. esculentum* allele (p ≠ p* ≠ e). This represents the most informative class of markers that allow straightforward estimation of the two distortion parameters (α and β) as well as the recombination frequency (r). Marker By-4 and *Aps1/Taq*I is of this category. The configuration of the cross reads like:



♀ (F_{1a}) ♂ (F_{1b})

Using the same parameter notation as above, we have the following table of gamete combinations

♀ \ ♂	β *ol-4 p**	$1-\beta$ *ol-4 e*
$\alpha(1-r)$ *Ol-4 p*	$\alpha\,\beta(1-r)$	$\alpha\,(1-\beta)(1-r)$
$(1-\alpha)r$ *Ol-4 e*	$(1-\alpha)\beta r$	$(1-\alpha)(1-\beta)r$
αr *ol-4 p*	$\alpha\,\beta r$	$\alpha(1-\beta)r$
$(1-\alpha)(1-r)$ *ol-4 e*	$(1-\alpha)\beta(1-r)$	$(1-\alpha)(1-\beta)(1-r)$

Thus, the two-way table of genotypes reads:

	Ol-4/ol-4	*ol-4/ol-4*	*Sum*
*p/p**	$\alpha\,\beta(1-r)$	$\boxed{\alpha\,\beta r}$	$\alpha\,\beta$
*e/p**	$\boxed{(1-\alpha)\beta r}$	$(1-\alpha)\beta(1-r)$	$(1-\alpha)\beta$
e/p	$\alpha\,(1-\beta)(1-r)$	$\boxed{\alpha(1-\beta)r}$	$\alpha(1-\beta)$
e/e	$\boxed{(1-\alpha)(1-\beta)r}$	$(1-\alpha)(1-\beta)(1-r)$	$(1-\alpha)(1-\beta)$

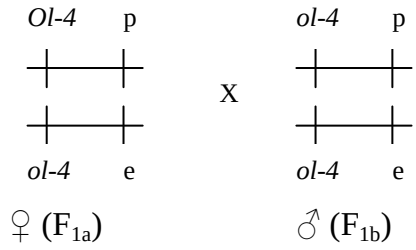
Notice that the boxed genotypes together represent a proportion *r* (independent of α , β). So *r* is estimated by counting these genotypes. Likewise α and β are estimated by adding the appropriate classes in the right margin of the above table (p/p* + e/p for α ; p/p* + e/p* for β) . Using the numbers given in Table 4 we obtain for

By-4 : $\hat{\alpha} = 0.479;\ \hat{\beta} = 0.823;\ \hat{r} = 0.0645$, and for

Aps1/Taq I: $\hat{\alpha} = 0.479;\ \hat{\beta} = 0.828;\ \hat{r} = 0.0538$.

We observe that the estimates for α and β are the same as the ones obtained for markers By-4/*Hyp*CH4IV and By-4/*Apo*I, which is not surprising since they represented the same locus (By-4).

(D) Markers that distinguish between the *L. peruvianum* and *L. esculentum* alleles, but not between the two *L. peruvianum* alleles (p* = p ≠ e). Marker *Aps1/Sau*96I is of this type. The configuration of the cross reads like:



From this we see that, considering the marker locus only, any segregation distortion at the marker cannot be ascribed to either parent or to both parents. In other words, estimation of the distortion parameters must go along with estimation of *r*, and vice versa. In this sense this marker category represents the most 'difficult' one: it requires simultaneous estimation of α, β and r.

Proceeding as before, using α and β for female and male transmission frequencies at the marker locus and *r* for recombination frequency, we obtain the following two-way table of genotypes (numbers in parentheses are observed numbers for marker *Aps1/Sau*96I in Table 4, **Chapter 4**).

	Ol-4 / ol-4	*ol-4 / ol-4*	Sum
p/p	$\alpha\beta(1-r)$ (71)	$\alpha\beta r$ (3)	$\alpha\beta$ (74)
e/p	$\alpha(1-\beta)(1-r) + (1-\alpha)\beta r$ (22)	$\alpha(1-\beta)r + (1-\alpha)\beta(1-r)$ (73)	$\alpha(1-\beta) + (1-\alpha)\beta$ (95)
e/e	$(1-\alpha)(1-\beta)r$ (0)	$(1-\alpha)(1-\beta)(1-r)$ (17)	$(1-\alpha)(1-\beta)$ (17)
Sum	(93)	(93)	1 (186)

For the simultaneous estimation of α , β, and *r* we proceed as follows. As can be seen from the table above, α and β can be estimated from the observed frequencies at the marker locus (probabilities in the right margin). We also see that for the marker genotype frequencies α and β are interchangeable, which means that, in case α and β are not equal, we cannot ascribe the estimate to either parent. However, we observe a 1 : 1 ratio at the *Ol-4* locus, which means that for a marker closely linked to *Ol-4* the value of α must be close to 0.5 (α is the female transmission rate: a clear deviation from 0.5 would 'drag' along the alleles at *Ol-4*). Estimates of α and β are obtained by solving the appropriate likelihood equations using probabilities and observed frequencies at the marker locus. The resulting quadratic equation yields two equivalent solutions, *i.e.*

$(\hat{\alpha}, \hat{\beta}) = (0.483, 0.823)$ and $(\hat{\alpha}, \hat{\beta}) = (0.823, 0.483)$.

We accept the first one, since α should be close to 0.5; it also is in close agreement with the estimates obtained for the other markers. Next, we substitute $(\alpha, \beta) = (0.483, 0.823)$ in the expressions for the probabilities in the body of the table and (numerically) solve the resulting likelihood equation for *r*. This yields $\hat{r} = 0.0394$. However, should we have used the 'mirror' estimates of $(\hat{\alpha}, \hat{\beta})$, i.e. $(\hat{\alpha}, \hat{\beta}) = (0.823, 0.483)$, the incorrect estimate of *r* would have been $\hat{r} = 0.287$. This, again, shows the necessity of carefully interpreting the joint segregation data in order to avoid wrong conclusions.

Using the obtained estimates we have calculated the corresponding LOD values for linkage. The table below summarizes the results.

Marker	$\hat{\alpha}$	$\hat{\beta}$	$\hat{r}$	LOD
Aps1/T*aq*I	0.479	0.828	0.054	39.1
Aps1/*Sau*96I	0.483	0.823	0.039	27.7
By-4	0.479	0.823	0.065	36.7
By-4/*Apo*I	0.479	-	0.065	36.7
By-4/*Hyp*CH4IV	-	0.823	-	-

Summary

Tomato powdery mildew caused by *Oidium neolycopersici* has become a globally important disease of tomato. Besides a few recently released resistant cultivars, all tomato cultivars are susceptible to *O. neolycopersici.* It is the main disease that is controlled by using agro-chemicals in protected tomato production in Europe. Therefore, resistance to *O. neolycopersici* is an important trait in tomato breeding. Promising sources of resistance to *O. neolycopersici* are available in different *Lycopersicon* and *Solanum* species (Lindhout 1994a, Mieslerová et al. 2000), but the genetics and the resistance mechanisms are largely unknown. The aim of our research is to understand the interaction between tomato (*Lycopersicon esculentum*) and tomato powdery mildew (*O. neolycopersici*), by using well defined plant materials that contain individual resistance genes in an identical genetic background and by using a range of *O. neolycopersici* isolates (races).

In this thesis, the genetic basis for several resistances against *O. neolycopersici* originating from wild *Lycopersicon* species was determined, and the corresponding resistance genes were mapped on the tomato genome. Subsequently, a set of near isogenic lines (NILs) was generated with the individual qualitative resistance genes (*Ol*-genes) in a genetic background of the susceptible *L. esculentum* cv. Moneymaker. These NILs represent well-defined plant materials and are very useful tools to study the interaction between tomato and *O. neolycopersici*. Using these NILs, the race-specificity of the resistance conferred by the *Ol*-genes was determined and the resistance mechanisms associated with different *Ol*-genes were studied microscopically.

The resistance to *O. neolycopersici* originating from *L. parviflorum* G1.1601 (**Chapter 2**) is controlled by three QTLs (quantitative trait loci). *Ol-qtl1* is on Chromosome 6 in the same region as the *Ol-1/Ol-3* locus and also *Ol-5* locus (**Chapter 5**), which are involved in a hypersensitive resistance response to *O. neolycopersici*. *Ol-qtl2* and *Ol-qtl3* are located on Chromosome 12, separated by 25 cM. *Ol-qtl2* maps in the vicinity of the *Lv* locus that confers resistance to another tomato powdery mildew species, *Leveillula taurica*. The three QTLs, jointly explaining 68% of the phenotypic variation, were validated by testing F_3 progenies.

L. peruvianum LA2172 is one of the wild accessions that are resistant to *O. neolycopersici*. Several difficulties that are often associated with genetic studies of *L. peruvianum,* like crossing barriers and multi-alleles per locus, were also encountered in our study aimed at mapping the *O. neolycopersici* resistance in *L. peruvianum* LA2172. Consequently, multi-allelic, single-locus markers were exploited to unfold the complexities such as multi-allelism and distorted segregation in a pseudo-F_2 population derived from

L. esculentum cv. Moneymaker x *L. peruvianum* LA2172. Finally, it was concluded that the resistance to *O. neolycopersici* in *L. peruvianum* LA2172 is governed by a single dominant gene, designated *Ol-4*, which maps on tomato Chromosome 6 (**Chapter 4**).

In addition to *Ol-4*, we mapped and characterized two other dominant *Ol*-genes (*Ol-5* and *Ol-6*) and fine-mapped the *Ol-1* locus (**Chapter 5**). All the dominant *Ol*-genes mapped so far are located in three genetic loci on tomato Chromosome 6. On the long arm, one locus contains two resistance genes *Ol-1* and *Ol-3* (originating from *L. hirsutum* G1.1560 and G1.1290, respectively) and the other locus harbors *Ol-5* (originating from *L. hirsutum* PI247087). On the short arm, one locus contains the two resistance genes *Ol-4* (originating from *L. peruvianum* LA2172) and *Ol-6* (unknown origin).

Simple PCR markers were generated (**Chapter 2, 3, 4** and **5**) to integrate linkage maps, to assign unknown linkage groups to tomato chromosomes and to accurately map the *Ol*-genes and *Ol*-qtls. They facilitated the alignment of genetic linkage maps across distantly related species for comparison of map positions of the *Ol*-genes and *Ol*-qtls. Moreover, they were used as diagnostic markers to monitor the presence of the *Ol*-genes and *Ol*-qtls in the development of NILs via MAS (marker assisted selection). Detailed information of these PCR markers is available at: http://www.dpw.wau.nl/pv/CAPStomato/.

Aiming at studying the interaction between tomato and *O. neolycopersici*, NILs were generated that contain individual *Ol*-genes in a genetic background of *L. esculentum* cv. Moneymaker (**Chapter 6**). In addition to the dominant *Ol*-genes, the recessive gene *ol-2* that originates from *L. esculentum* var *cerasiforme* and is located on chromosome 4 (Ciccarese et al. 1998, De Giovanni et al. 2004) was also included in our study. The race-specificity of resistance to *O. neolycopersici* conferred by the *Ol*-genes was revealed by testing the NILs with local isolates in different geographic locations. Moreover, resistance mechanisms of different *Ol*-genes were described microscopically by using the NILs. The mechanism of resistance conferred by the dominant *Ol*-genes was associated with an HR, while the mechanism of resistance governed by the recessive gene *ol-2* was associated with papillae formation (**Chapter 6**). Our study provides more insight in the host-pathogen interaction between *Ol*-genes and *O. neolycopersici* isolates, and facilitates the breeding of tomato cultivars for resistance to *O. neolycopersici*.

Samenvatting

Echte meeldauw op tomaat, veroorzaakt door *Oidium neolycopersici*, is wereldwijd een belangrijke ziekte van dit gewas. Met uitzondering van een aantal recent op de markt gebrachte cultivars, zijn praktisch alle tomatenrassen vatbaar voor *O. neolycopersici*. In de beschermde tomatenteelt in Europa is dit de voornaamste ziekte die nog met chemische bestrijdingsmiddelen wordt bestreden. Bovenstaande geeft aan dat resistentie tegen *O. neolycopersici* een belangrijke eigenschap is voor de tomatenveredeling. In verscheidene *Lycopersicon* en *Solanum* soorten zijn veelbelovende resistenties tegen *O. neolycopersici* gevonden (Lindhout 1994a, Mieslerova *et al*. 2000). Zowel de mechanismen als de genetische basis van deze resistenties zijn nagenoeg onbekend. Het doel van het hier beschreven onderzoek is om de interactie tussen tomaat (*Lycopersicon esculentum*) en de echte meeldauw van tomaat beter te begrijpen, daarbij gebruikmakend van goed gedefinieerd plantmateriaal, dat verschillende resistentiegenen bevat in een identieke genetische achtergrond en van een sortiment *O. neolycopersici* isolaten.

In dit proefschrift wordt de genetische basis achterhaald van een aantal resistenties tegen *O. neolycopersici*, afkomstig uit wilde *Lycopersicon* soorten, en worden de corresponderende resistentiegenen gekarteerd op het tomatengenoom. Vervolgens is een reeks Bijna Isogene Lijnen (Near Isogenic Lines, afgekort als NILs) gemaakt, elk met een individueel kwalitatief resistentie gen (*Ol*-gen), in de genetische achtergrond van de vatbare *L. esculentum* cv. ‘Moneymaker’. Deze serie NILs vertegenwoordigt een verzameling goed gedefinieerd plantmateriaal, en vormt als zodanig een belangrijk gereedschap om de interactie tussen tomaat en *O. neolycopersici* te bestuderen. De isolaat-specificiteit van de verschillende *Ol*-resistentiegenen is vastgesteld, en de hiermee overeenkomende resistentiemechanismen zijn microscopisch bestudeerd.

De resistentie tegen *O. neolycopersici* afkomstig uit *L. parviflorum* G1.1601 (**Hoofdstuk 2**) wordt gereguleerd door drie QTLs (Quantitative Trait Locus = plaats op het genoom coderend voor een kwantitatieve eigenschap). *Ol-qtl1* ligt op chromosoom 6, in hetzelfde gebied als de *Ol-1/Ol-3* locus en de *Ol-5* locus (**Hoofdstuk 5**). Deze drie monogeen dominante resistentiegenen (*Ol*-genen) veroorzaken een overgevoeligheidsreactie na infectie van tomaat met *O. neolycopersici. Ol-qtl2* en *Ol-qtl3* liggen op chromosoom 12, van elkaar gescheiden door 25 centimorgan. *Ol-qtl2* is gekarteerd nabij de locus van het *Lv*-gen, hetwelk resistentie verleent tegen een andere echte meeldauw van tomaat, namelijk *Leveilula taurica*. Gezamenlijk verklaren de drie QTLs achtenzestig procent van de fenotypische variatie, hetgeen gecontroleerd en bevestigd is in resistentietoetsen met F_3 nakomelingschappen.

L. peruvianum LA2172 is een andere wilde accessie met resistentie tegen *O. neolycopersici*. Genetische studies aan *L. peruvianum* worden veelal bemoeilijkt door kruisingsbarrières en het voorkomen van meerdere allelen per genetische locus. Ook onze studie, gericht op de kartering van *O. neolycopersici* resistentie in *L. peruvianum* LA2172, werd bemoeilijkt door bovengenoemde factoren. Teneinde de complexiteit van het overervingspatroon, veroorzaakt door multiple allelie en scheve uitsplitsing in een pseudo F2 populatie van de kruising L. esculentum cv. 'Moneymaker' × L. peruvianum LA2172 te kunnen ontrafelen werden locus-specifieke multi-allelische merkers ontwikkeld. Uiteindelijk kon worden geconcludeerd dat de resistentie tegen *O. neolycopersici* in *L. peruvianum* LA2172 veroorzaakt wordt door een enkel dominant gen, genoemd *Ol-4*, gelegen op chromosoom 6 van tomaat (**Hoofdstuk 4**).

Naast *Ol-4* hebben we twee andere dominante *Ol*-genen (*Ol-5* en *Ol-6*) gekarteerd en gekarakteriseerd en is de *Ol-1* locus nauwkeurig gekarteerd (**Hoofdstuk 5**). Al de tot nu toe gekarteerde *Ol*-genen liggen in drie genetische loci op chromosoom 6 van tomaat. Op de lange arm van chromosoom 6 ligt een locus met de twee resistentiegenen *Ol-1* en *Ol-3* (respectievelijk afkomstig uit *L. hirsutum* G1.1560 en G1.1290) en een andere locus met *Ol-5* (afkomstig uit *L. hirsutum* PI247087). Op de korte arm is een locus gekarteerd met de twee resistentiegenen *Ol-4* (geïntroduceerd uit *L. peruvianum* LA1272) en *Ol-6* van onbekende herkomst.

Om de verschillende genetische kaarten met elkaar te integreren, om onbekende koppelingsgroepen aan bekende tomatenchromosomen te kunnen toewijzen én om de *Ol*-genen en de *Ol*-QTLs trefzeker te kunnen karteren zijn eenvoudige PCR-merkers gemaakt. Deze merkers maakten het mogelijk om genetische kaarten van kruisingen met ver verwante soorten met elkaar in overeenstemming te brengen om zodoende de kaartpositie van de *Ol*-genen en *Ol*-QTLs met elkaar te kunnen vergelijken. Bovendien werden deze PCR merkers toegepast als eenvoudige diagnostische merkers om de aanwezigheid van *Ol*-genen en *Ol*-QTLs te registreren tijdens het ontwikkelen van de NILs (dit is merker-gestuurde selectie). Gedetailleerde informatie omtrent deze PCR-merkers is te vinden op de website: http://www.dpw.wau.nl/pv/CAPStomato/.

De NILs met individuele *Ol*-genen in een genetische achtergrond van *L. esculentum* cv. 'Moneymaker' zijn gemaakt met als doel de interactie tussen tomaat en *O. neolycopersici* te kunnen bestuderen (**Hoofdstuk 6**). Naast de dominante *Ol*-genen, is ook het recessieve resistentiegen *ol-2* meegenomen in deze studie; *ol-2* is afkomstig uit *L. esculentum* var. *cerasiforme* en gekarteerd op chromosoom 4 (Ciccarese *et al*., 1998; De Giovanni *et al*., 2004). De isolaat-specificiteit van de *Ol*-genen, welke resistentie verlenen tegen *O. neolycopersici*, is opgehelderd door de NILs te testen met lokale isolaten afkomstig uit verschillende gebieden

van over de hele wereld. Daarnaast is het resistentiemechanisme van de verschillende *Ol*-genen beschreven na microscopische analyses van geïnfecteerde NILs. Terwijl het resistentiemechanisme verleend door de dominante *Ol*-genen altijd was geassocieerd met een overgevoeligheidsreactie (HR), ging de resistentie verleend door het recessieve *ol-2* gen gepaard met de vorming van pappillen (**Hoofdstuk 6**).

Deze studie verschaft meer inzicht in de plant-pathogeen interactie tussen de *Ol*-genen van tomaat en de verschillende *O. neolycopersici* isolaten. Daarnaast vergemakkelijken de ontworpen PCR-merkers het kweken van tomatenrassen die resistent zijn tegen *O. neolycopersici*.

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Yuling Bai was born on the 24th July, 1964 in Beiyang county, Henan Province, China. After finishing high school in 1981, she studied at Henan Agricultural University (HAU, China) and received a BSc degree in 'Agronomy' in 1985 and a MSc degree in 'Plant Genetics and Breeding' in 1988. After graduation, she worked in the Department of Horticulture at HAU as a lecturer for several years (from 1988) and then became an associate professor in 1995. In October 1997, she came to Wageningen University (WU) as a visiting scholar, working on the project of 'Molecular characterization of avirulence gene 1 from *Fusarium oxysporum* f.sp. *lycopersici* and the corresponding resistance gene *I-1* from tomato (*Lycopersicon esculentum*)'. Meanwhile, she followed MSc courses of 'Biotechnology' at WU and got her second MSc degree in 'Biotechnology' with distinction in 2000. From 2000 to 2004, she was a research assistant (AIO) in the Laboratory of Plant Breeding at WU on the project of 'On the origin of tomato powdery mildew species and the corresponding gene in the genus *Lycopersicon'*. After getting her PhD, she will continue her research in the same project as a post-doctor.