Tomato Defense to *Oidium neolycopersici*: Dominant *Ol* Genes Confer Isolate-Dependent Resistance Via a Different Mechanism Than Recessive *ol-2*

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Tomato powdery mildew caused by *Oidium neolycopersici* has become a globally important disease of tomato (*Lycopersicon esculentum*). To study the defense responses of tomato triggered by tomato powdery mildew, we first mapped a set of resistance genes to *O. neolycopersici* from related *Lycopersicon* species. An integrated genetic map was generated showing that all the dominant resistance genes (*Ol-1*, *Ol-3*, *Ol-4*, *Ol-5*, and *Ol-6*) are located on tomato chromosome 6 and are organized in three genetic loci. Then, near-isogenic lines (NIL) were produced that contain the different dominant *Ol* genes in a *L. esculentum* genetic background. These NIL were used in disease tests with local isolates of *O. neolycopersici* in different geographic locations, demonstrating that the resistance conferred by different *Ol* genes was isolate-dependent and, hence, may be race-specific. In addition, the resistance mechanism was analyzed histologically. The mechanism of resistance conferred by the dominant *Ol* genes was associated with hypersensitive response, which varies in details depending on the *Ol*-gene in the NIL, while the mechanism of resistance governed by the recessive gene *ol-2* on tomato chromosome 4 was associated with papillae formation.

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 *Oidium lycopersicum* (later changed to *O. lycopersici*) for the European tomato powdery mildew fungus that appeared to originate from Australia. However, the pathogen that caused the recent outbreaks of tomato powdery mildew outside Australia was recently renamed *O. neolycopersici*. Based on morphological features of the fungus and the internal transcribed spacer sequence of the ribosomal DNA, it differs from *O. lycopersici* (Jones et al. 2001; Kiss et al. 2001). Curiously, the causal agent of tomato powdery mildew in Australia, *O. lycopersici*, has not been found on any tomato outside Australia (Kiss et al. 2001, in press).

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The initial events of colonization of susceptible tomato by *O. neolycopersici* have been described by Jones and associates (2000). Morphologically, *O. neolycopersici* can easily be distinguished from another species causing tomato powdery mildew, *Leveillula taurica*, which occurs in subtropical regions. The mycelium of *L. taurica* grows into the mesophyll of the leaf and is visible on the under side of the leaf, while *O. neolycopersici* mainly grows on the upper side and usually does not penetrate the mesophyll (Lindhout et al. 1994a).

The cultivated tomato is susceptible, but resistance to *O. neolycopersici* has been observed in wild *Lycopersicon* species (Lindhout et al. 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 et al. 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). Little is known about the genetic variation within *O. neolycopersici*. A set of *Lycopersicon* accessions showed differential reactions with isolates originating from the Czech Republic, Germany, The Netherlands, and Great Britain (Lebeda and Mieslerová 2000). Recently, Kashimoto and associates (2003) reported that one resistant tomato cultivar bred in The Netherlands is susceptible to the Japanese *O. neolycopersici* isolate KTP-01. These results suggested 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. So far, several monogenic and polygenic genes conferring resistance to *O. neolycopersici* have been studied in tomato. The dominant genes *Ol-1* and *Ol-3* (originating from *Lycopersicon hirsutum* G1.1560 and G1.1290, respectively) have been mapped on the long arm of tomato chromosome 6 (Huang et al. 2000b; Lindhout et al. 1994b). Another dominant gene, *Ol-4* (originating 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 (QTL) have been identified governing the resistance to *O. neolycopersici* in *L. parviflorum* G1.1601. One QTL (*Ol-qt1*) interval overlaps with *Ol-1* and *Ol-3*, and two other linked QTL (*Ol-qt2* and *Ol-qt3*) are located on chromosome 12 in the vicinity of the *Lv* gene that confers resistance to *L. taurica* (Bai et al. 2003). In addition, a recessive resistance gene (*ol-2*)

has been reported in *L. esculentum* var. *cerasiforme* (Ciccarese et al. 1998), located on chromosome 4 (De Giovanni et al. 2004). Finally, a very high level of resistance to *O. neolyopersici* was identified in *L. hirsutum* PI247087 (Latterot et al. 1995), and preliminary results showed that this resistance was probably polygenic, while the locus with major effect seemed to be linked to *Ol-1* (Moretti and Caranta 2000).

In plants, resistance genes occur in different genomic arrangements. The simplest arrangement is a locus consisting of a single resistance gene that can show 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 races (Islam and Shepherd 1991). More frequently, resistance genes are members of multigene families and are 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, in which homologous genes are widely dispersed over a chromosome arm, like the *Dm3* cluster in lettuce, in which 24 homologues of *Dm3* are dispersed over the genetic region surrounding *Dm3*, spanning at least 3.5 Mb (Meyers et al. 1998).

In this study, we aimed at characterizing the genome organization of *Ol* genes in more detail and at studying the interaction between the *Ol* genes in tomato and different *O. neolyopersici* isolates. Therefore, we fine-mapped *Ol-1* and *Ol-4* and, in addition, we characterized the resistance (named *Ol-5*)

originating from *L. hirsutum* PI247087 and a new resistance (named *Ol-6*) from an advanced breeding line (ABL). By repeated backcrossing, individual *Ol* genes were introgressed into the genetic background of *L. esculentum* cv. Moneymaker to generate near-isogenic lines (NIL). By using these NIL, race specificities and resistance mechanisms associated with the *Ol* genes were studied.

RESULTS

Five *Ol* genes are organized in three loci on tomato chromosome 6.

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 *L. esculentum* cv. Moneymaker (MM) $\times$ *L. hirsutum* G1.1560 (Huang et al. 2000a). 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 (BSA). The R pool was homozygous for *L. hirsutum* alleles and the S pool was homozygous for *L. esculentum* alleles in an interval between the restriction fragment length polymorphism (RFLP) markers TG153 and TG164 including the *Ol-1* locus (Fig. 1A). By using 400 *Pst*I/*Mse*I amplified fragment length polymorphism (AFLP) primer combinations, 62 candidate AFLP markers were identified. In a test panel (Table 1), 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, P1349 and P2147, were most closely linked to *Ol-1*.

Fig. 1. Genetic maps for part of chromosome 6 showing map positions of *Ol* genes based on different populations. **A**, Skeleton map showing map positions of *Ol-1* relative to restriction fragment length polymorphism markers and a polymerase chain reaction (PCR) marker (SCAF10) retrieved by Huang and associates (2000a). **B**, *Ol-1* fine-mapping position, using a BC_1S_1 population of *Lycopersicon esculentum* cv. Moneymaker (MM) $\times$ 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 $\times$ *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 $\times$ ABL. **E**, Skeleton map for tomato chromosome 6 showing relative positions of the mapped *Ol* genes and *Ol-qt1* (Bai et al. 2003). The map position of centromere is indicated between markers GP79L and Aps1, according to Figure 1A (Huang et al. 2000a). Bars indicate the quantitative trait loci (QTL) intervals for which the inner gray bar shows a 1-LOD (log of the likelihood) support interval; the outer bar (dotted line) shows a two-LOD support interval. Simple PCR markers are used for B through E.

To facilitate recombinant screening in the region flanked by TG25 and H9A11, RFLP probes (TG25 and H9A11) and the two AFLP markers (P1349 and P2147) were sequenced and converted into simple polymerase chain reaction (PCR) markers (Table 2). One BC₁S₁ population of 1,413 plants derived from the cross of MM × ABL-*Ol-1* (Table 1) was screened for recombinants between PCR markers tg25 and H9A11, and 17 recombinants were identified. In this population, the estimated genetic distance between these two markers was 0.64 cM, which is approximately 10 times lower than the distance in the F₂ population from an interspecific cross (Fig. 1A) (Huang et al. 2000a). The 17 BC₁S₁ recombinants were selfed to produce BC₁S₂ lines that were subsequently tested for disease resistance and were screened for markers in the region flanked by TG25 and H9A11. One recombinant (1 in 17) showed that *Ol-1* is below PCR marker P1349 and another recombinant was found between *Ol-1* and H9A11. Two recombinants in the test panel (Table 1) showed that P1349 is distal to SCARF10, the closely linked marker proximal to *Ol-1*, as reported by Huang and associates (2000a) (Fig. 1A). Thus, *Ol-1* is fine-mapped between PCR markers P1349 and H9A11 on the long arm of chromosome 6 (Fig. 1B). As the recombinants were obtained from different “source populations” (the ABL-*Ol-1*-derived BC₁S₁ population and the interspecific F₂ population; Table 1) with large differences in recombination frequencies, the estimated genetic distance between *Ol-1* and the closely linked markers also varied considerably. On the basis of the ABL-*Ol-1*-derived populations, the distance between *Ol-1* and P1349/P2147 was 0.035 cM (1/2,826 gametes), while in the interspecific F₂ population, this distance would be 10 times higher.

Fine-mapping of *Ol-4*. *Ol-4* has previously been mapped above PCR marker *Aps1* on tomato chromosome 6 by using a pseudo-F₂ population of MM × *L. peruvianum* LA2172. Further fine-mapping of *Ol-4* in this pseudo-F₂ population was complicated as multi-allelism at a single locus occurred (Bai et al.

2004b). To reduce the number of segregating alleles at the same locus, a BC₂S₁ population of MM × LA2172 (*n* = 112; Table 1) was generated, which was used to determine the segregation of the resistance and chromosome 6-specific PCR markers (Table 2). No recombinants were found between *Ol-4* and PCR markers GP79L and 32.5Cla (Fig. 1C), and this was confirmed in BC₃S₁ populations of MM × LA2172 (96 BC₃S₁ plants in total). On the short arm of tomato chromosome 6, two GP79L loci are present (Kaloshian et al. 1998). One is in an introgressed region of approximately 650 kb containing the *Mi* gene from *L. peruvianum*; the other is distal to this region. The 32.5Cla locus maps in the 650-kb *Mi-1* introgressed region on the short arm (Liharska 1998). As the *Ol-4* locus was completely linked to GP79L and 32.5Cla, the *Ol-4* gene is most likely located on the short arm of chromosome 6 (Fig. 1C).

Mapping of *Ol-5*. One advanced breeding line (ABL₁) obtained from INRA (Montfavet, France) harbors resistance to *O. neolyopersici* originating from *L. hirsutum* PI247087. A preliminary study of Moretti and Caranta (2000) indicated that this resistance is polygenic but with a major gene near *Ol-1*. Our aim was to determine the inheritance of this resistance and map the major gene for resistance. Therefore, a disease test was applied on three BC₂ families of the cross MM × ABL₁ (Table 1). The segregation of R to S plants jointly as well as in each BC₂ family fitted a segregation ratio of 1:1 for a monogenic dominant trait, suggesting that one dominant gene (designated as *Ol-5*) governed the resistance in ABL₁. To map *Ol-5*, a set of codominant PCR markers on tomato chromosome 6 (Table 2) was scored in a BC₂S₁ population (*n* = 100; Table 1). A genetic linkage map was constructed, which positioned *Ol-5* between PCR markers tg25 and P2147 on chromosome 6 (Fig. 1D). A comparison of the *Ol-5* map with the *Ol-1* map showed that orders of marker loci on both maps were identical. As *Ol-5* maps proximal to the PCR marker P2147, we conclude that *Ol-5* represents another *Ol* locus on the long arm of chromosome 6, about 1 cM proximal of the *Ol-1* locus (Fig. 1E).

Table 1. Plant materials and summary of the disease tests

<i>Ol</i> -genes	Purpose	Population	Original cross	Disease test	
				Susceptible and resistant control	Evaluation ^w
<i>Ol-1</i>	Bulked segregant analysis	F ₂	<i>Lycopersicon esculentum</i> cv. Moneymaker (MM) × <i>L. hirsutum</i> G1.1560 ^x	20 MM and 20 ABL- <i>Ol-1</i> ^y plants in the disease test of recombinant inbred lines	Used DI of 0 to 3 at 11 and 13 dpi in the disease test of recombinant inbred lines
	To position markers linked to <i>Ol-1</i>	F ₂ recombinants (a test panel ^z)			
	Recombinant screening	BC ₁ S ₁ (<i>n</i> = 1413)	MM × ABL- <i>Ol-1</i>		
	Fine-mapping	17 recombinant inbred lines (BC ₁ S ₂), 16 plants per line			
<i>Ol-4</i>	Fine-mapping	BC ₂ S ₁ (<i>n</i> = 112)	MM × <i>L. peruvianum</i> LA2172	20 MM and 20 LA2172 plants	Scored plants as R or S at 9 and 12 dpi.
<i>Ol-5</i>	Inheritance study and mapping	Three BC ₂ (<i>n</i> = 79, in total) and one BC ₂ S ₁ (<i>n</i> = 100)	MM × ABL ₁	20 MM and 20 ABL ₁ plants	Used DI at 11 and 13 dpi.
<i>Ol-6</i>	Inheritance study and mapping	Two BC ₂ (<i>n</i> = 52 total), one BC ₃ S ₁ (<i>n</i> = 80)	MM × ABL ₂	20 MM and 20 LA2172 plants (disease test for BC ₂); 10 MM and 10 LA2172 plants (disease test of BC ₃ S ₁)	Scored plants as R or S at 11 and 13 dpi for disease test on BC ₂ , and at 13 and 15 dpi for disease test on BC ₃ S ₁

^wDI = disease index, R = resistant, and S = susceptible.

^x This F₂ population was tested for resistance to *Oidium neolyopersici* and screened for 15 RFLP markers specific for chromosome 6 and four polymerase chain reaction markers (Huang et al. 2000a).

^y ABL = advanced breeding line.

^z A set of 16 informative genotypes is selected as a test panel. These are recombinants in the interval between restriction fragment length polymorphism (RFLP) markers TG153 and TG164 and provide conclusive evidence for markers inside the interval between markers TG25 and H9A11.

Mapping of Ol-6. ABL₂ harbors resistance to *O. neolyopersici*, but the origin of this resistance is unknown, as it has been found in a breeding line of unknown ancestry. The inheritance of the resistance in ABL₂ was studied by applying a disease test on two BC₂ families (Table 1). In total, the two BC₂ families showed 26 R and 26 S plants, fitting a monogenic model, which was confirmed in a BC₃S₁ population ($n = 80$, $\chi^2 = 0.02$, $P = 0.90$). Thus, the complete resistance in ABL₂ is conferred by one dominant gene, designated as *Ol-6*.

BSA was performed to find molecular markers linked to *Ol-6*. Six AFLP markers were obtained by using 64 *Pst*I/*Mse*I AFLP primer combinations, and one of the AFLP markers (P1449) was converted into a simple PCR marker. Remarkably, this marker was also mapped on chromosome 6 by using a reference F₂ population of MM × *L. pennellii* LA716 (Haanstra et al. 1999b). The cosegregation between AFLP marker P1449 and its derived PCR marker could not be verified as no polymorphisms were found for this locus between MM and ABL₂ after screening with 24 restriction enzymes (Bai et al. 2004a). Another *Ol-6*-cosegregating AFLP marker, P1346, was converted into a dCAPS (derived cleaved amplified polymorphic sequence) marker (Neff et al. 1998) and was designated dP1346 (Table 2). This marker was located 4 cM distal to 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 PCR markers used to map *Ol-4* was applied in the BC₃S₁ population segregating for *Ol-6*. In this mapping population, *Ol-6* fully cosegregated with GP79L and 32.5Cla that were mapped 2 cM apart from dP1346. Interestingly, ABL₂ showed a phenotype of *L. peruvianum* LA2172 for PCR markers on the short arm of chromosome 6 but a MM marker phenotype for PCR

markers on the long arm of chromosome 6, indicating that ABL₂ likely contains a short introgressed region confined to the short arm of chromosome 6. As *Ol-4* and *Ol-6* map to the same locus and the origin of *Ol-6* is unknown, the possibility that *Ol-6* is identical to *Ol-4* cannot be excluded.

Development of NIL.

To develop NIL that only differ for *Ol* genes, resistant donor accessions were crossed with MM. Homozygous BC₃S₁ plants (R and S) of these crosses were selected (discussed below) and subjected to AFLP analysis in order to compare their genetic background with the recurrent parent MM. Most BC₃S₁ plants were genetically more than 90% similar to MM. 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,

Table 3. Local isolates of *Oidium neolyopersici* and disease test locations

Name ^y	Local isolate name	Test locations
On-Ne	...	Wageningen University, Wageningen, The Netherlands
On-Hu	BP-P5	Plant Protection Institute of the Hungarian Academy of Sciences, Budapest, Hungary
On-Cz	Isolate 1/98	Palacky University, Olomouc, Czech Republic
On-It	... ^z	Nunhems Seeds, Bologna, Italy
On-Fr (A)	76 local	Rijk Zwaan, Avignon, France
On-Fr (B)	...	De Ruiter Seeds, Avignon, France
On-Fl	...	University of Florida, Bradenton, U.S.A.

^y Isolate name used in this study.

^z Obtained from University of Perugia.

Table 2. Primer sequences, lengths of polymerase chain reaction (PCR) products and enzymes revealing a polymorphism for CAPS/SCAR markers^a

Marker name	Marker type ^y / original type	Primer name ^w	Primer sequence (5' - 3') ^x	Tm (°C) ^y	PCR product size (bp)	Polymorphism between <i>Lycopersicon esculentum</i> cv. Moneymaker and			
						<i>L. hirsutum</i> G1.1560	<i>L. peruvianum</i> LA2172	ABL ₁	ABL ₂
dP1346	dCAPS/AFLP	dP1346-F dP1346-R	ttggaatcaagaaaagggttaa atgactatgtccataaatgaa	47	200	nd ^z	<i>Hinc</i> II	nd	<i>Hinc</i> II
GP79L	CAPS/RFLP	GP79L-F GP79L-R	cactcaatgggggaagcaac aatggtaaagcagcgggact	56	2,000	<i>Apo</i> I	<i>Apo</i> I	<i>Apo</i> I	<i>Apo</i> I
32.5Cla	CAPS/RFLP	32.5Cla-F 32.5Cla-R	acacgaaacaaagtgcgaag ccaccaccaaacaggagtgtg	56	773	<i>Hinf</i> I	<i>Hinf</i> I	nd	<i>Hinf</i> I
<i>Aps</i> I	CAPS/RFLP	<i>Aps</i> I-F <i>Aps</i> I-R	atggtgggtccaggttataag cagaatgagcttctgcgaatc	56	1,000	<i>Dde</i> I	<i>Sau</i> 96I	<i>Hinf</i> I	nd
P2147	CAPS/AFLP	P2147-F1 P2147-R1	taacaatctcgaccatagtcc ccatacccgaaatttctctcc	56	300	<i>Dde</i> I	<i>Dde</i> I	<i>Hae</i> III	nd
P1349	CAPS/AFLP	P1349-F P1349-R	tgctaagaatcagaaccacacct acaacaagctgacccactaaaga	56	500	<i>Xcm</i> I	nd	<i>Xcm</i> I	nd
ct184	CAPS/RFLP	ct184-F ct184-R	ttccgtgtattgccaacaa accaaagatcaatggatgg	56	298	<i>Dde</i> I	<i>Dde</i> I	<i>Dde</i> I	nd
tg25	Codominant SCAR/RFLP	tg25-conF tg25-prR1	taatttggcactgccgt ttgtyatrtgtgtytatcg	52	350 + 300	No SCAR	SCAR	SCAR	nd
tg25	Codominant SCAR/RFLP	tg25-conF tg25-ehR1	taatttggcactgccgt catgttgygyttatcraaagtc	52	350 + 300	SCAR	No SCAR	SCAR	nd
H9A11	Codominant SCAR/RFLP	H9A11-conF H9A11-conR	tgctctaacaaaatcaccaaaatc aaatgggtcaacaaagtctattgag	52	450 + 400	SCA?R	SCAR	SCAR	nd
ct174	CAPS/RFLP	ct174-F2 ct174-R1	aaaagaagcgcacaaatagacc ggctctcccatacaacagtc	56	500	<i>Hae</i> III	<i>Hae</i> III	<i>Hae</i> III	nd

^a The information will be maintained and updated regularly on the Wageningen University website.

^y CAPS, cleaved amplified polymorphic sequence; dCAPS, derived CAPS; SCAR, sequence characterized amplified region.

^w F, forward primer; R, reverse primer.

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

^y PCR annealing temperature.

^z nd, not subjected to restriction enzyme screening.

only three were present in the BC₃S₁ plants containing *Ol-1*. Two fully cosegregated with the resistance, indicating that these two markers were located on the *Ol-1* introgression, while the third marker did not cosegregate with *Ol-1*. Similar results were obtained for the BC₃S₁ plants containing *Ol-3*, *Ol-5*, and *Ol-6* genes. In contrast, BC₃S₁ plants containing the *Ol-4* gene showed that 11 of 48 AFLP marker alleles from the donor parent still segregated in these BC₃S₁ plants. This result can be explained by the fact that these BC₃S₁ plants were derived from an interspecific cross between MM and *L. peruvianum*, while the others were derived from ABL plants that were already genetically similar to MM. BC₃S₂ lines from selfed homozygously resistant BC₃S₁ plants are referred to as *Ol* NIL and are further characterized in this study, together with the line F3-*ol-2* (discussed below).

Race specificities of resistance conferred by the *Ol* genes.

Initially, we considered testing the NIL in one location with isolates collected from different parts of the world, in order to investigate the interactions between the NIL and the *O. neolyopersici* isolates. However, we previously experienced cross contamination of different powdery mildews during maintenance and propagation, even though spore-proof growth cabinets were used. Therefore, we tested the NIL with local isolates in different geographic locations (Table 3). To reduce the variation due to different experimental conditions and observers, the test conditions were standardized and detailed descriptions with pictures of symptoms for disease evaluation were provided to all test sites. Moreover, the NIL were coded to promote unbiased evaluation. At each location, the variation in disease index (DI) among the tested plants within each line was small, indicating that the results were reliable. A mean DI was calculated for each line (discussed below).

MM was susceptible (DI = 2) to all the isolates tested. NIL *Ol-1* and *Ol-5* were incompletely resistant (1 < DI < 2) to the Hungarian isolate *On-Hu* and were completely resistant (DI = 1) to all other isolates tested. F3-*ol-2* and NIL *Ol-3* were completely resistant to all isolates, except in Italy where, in one test, F3-*ol-2* was completely resistant to the Italian isolate *On-It* and 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 isolates but one, the Czech isolate *On-Cz*. The susceptibility of NIL *Ol-4* and *Ol-6* to *On-Cz* was similar to the susceptible control MM and was confirmed by a repeated disease test. In conclusion, our data showed a clear difference in isolate-*Ol* NIL interaction, suggesting gene-for-gene interactions in the tomato-*O. neolyopersici* pathosystem.

Different resistance mechanisms associated with the *Ol* genes.

Resistance mechanisms were studied using the *O. neolyopersici* isolate *On-Ne*. To obtain good preparations for microscopic observation, a pilot experiment was performed with two inoculation methods (discussed below). 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 less reliable. The spray-inoculation gave an equal and dense spore distribution, allowing sufficient numbers of infection units (IU) to be evaluated. In MM, IU were well established at 65 h postinoculation (hpi), showing branching hyphae and primary and secondary haustoria. Thus, for the experiment with NIL, the spray-inoculation method was used, and microscopic observations were done on samples collected at 65 hpi.

To verify the effectiveness of the resistance conferred by these *Ol* genes at a high spore concentration (3×10^5 conidia per milliliter, 15 times higher than normal), sporulation was recorded on two plants per line. Susceptible control MM plants sporulated heavily (DI = 3), while the NIL containing the genes of *Ol-1*, *Ol-3*, *Ol-4*, and *Ol-5* showed similar resistance levels as found in disease tests with a normal inoculum density (2×10^4 conidia per milliliter). The F3-*ol-2* and NIL *Ol-6* showed some fungal spots surrounded by necrosis (DI = 1), compared with no-symptoms (DI = 0) upon a spore density of 2×10^4 conidia per milliliter. Therefore, we concluded that the resistance in the NIL remained effective also at higher inoculum pressure.

The fungal development was microscopically evaluated (Table 4). On all lines, germ tubes had formed primary appressoria at 65 hpi. Compared with MM, NIL 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. 2A) became necrotic and only 2% of the cells with secondary haustoria showed the necrosis that is typical for the HR. In contrast, 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*. Two types of necrotic cells were observed. The first type (type 1) showed a fully shriveled epidermal cell with a shriveled haustorium (Fig. 2B); the second type (type 2) showed an epidermal cell with many particles and a normal or shriveled haustorium (Fig. 2C). In NIL *Ol-1* and *Ol-3*, types 1 and 2 were about equally mixed, but in NIL *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. 2B) that was associated with an almost complete arrest of further fungal development (Table 4).

Table 4. Development of *Oidium neolyopersici* isolate *On-Ne* on *Lycopersicon esculentum* cv. Moneymaker, *Ol*-NILs and F3-*ol-2* line^x

Genotype	Primary HS per IU ^{y,z}	Necrotic cells per HS ^y		Papilla per AP ^y		Papilla per HS ^y		No. per IU		
		Primary	Secondary	Primary	Secondary	Primary	Secondary	Hyphae	AP	Secondary HS
Moneymaker	93 a	0 a	2 a	0	0	0	0	4.9 a	4.9 a	2.5 a
NIL- <i>Ol-1</i>	86 a	32 b	25 b	0	0	0	0	3.8 b	4.6 a	2.2 ab
NIL- <i>Ol-3</i>	84 a	33 b	27 b	0	0	0	0	3.4 b	4.4 a	2.5 a
NIL- <i>Ol-5</i>	91 a	31 b	24 b	0	0	0	0	4.0 b	4.1 a	1.8 b
NIL- <i>Ol-6</i>	93 a	81 c	54 c	0	0	0	0	2.3 c	3.5 a	1.7 b
NIL- <i>Ol-4</i>	56 b	100 d	100 d	0	0	0	0	0.3 c	0.3 b	0.2 c
F3- <i>ol-2</i>	42 b	0 a	0 a	67	49	90	94	1.4 d	1.5 b	0.3 c

^x 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.

^y Numbers represent percentages.

^z IU = infection unit; HS=haustorium; AP = appressorium.

Remarkably, only in F3-*ol-2*, papilla formation was observed beneath the appressoria while HR was completely absent (Table 4; Fig. 2D). In this line, a lower number of IU (about 10 IU per square centimeter) was observed than in the other NIL (about 30 IU per square centimeter), which suggests inhibited spore germination. Further fungal growth was significantly reduced (Table 4). More than 90% of the haustoria were associated with papilla formation around the haustorial neck (Fig. 2E). From this experiment, it is not clear whether the papillae were penetrated by the fungus to produce a haustorium or the papilla material had been deposited around the haustorial neck after haustorium formation. Also it was not possible to determine whether the papillae were the cause of haustorium formation failure (Fig. 2D) or whether they were formed after the haustorium formation had been aborted due to another mechanism.

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. First, BSA was performed in different mapping populations to identify AFLP markers linked to the *Ol* genes. Subsequently, the linked AFLP markers were converted into simple diagnostic PCR markers, which were used as chromosomal anchors in a reference mapping population. After this, more RFLP in the corresponding chromosomal region were selected for conversion into 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 map positions of different *Ol* genes. This experimental approach allowed us to generate an integrated map comprising DNA markers and different *Ol* genes, which revealed the relative locations of five *Ol* genes that are organized in three genetic loci on tomato chromosome 6. On the long arm, one locus contains the gene *Ol-5* and the other locus harbors the genes *Ol-1* and *Ol-3*. As *Ol-1* and *Ol-3* are assumed to be allelic (Huang et al. 2000b), only *Ol-1* was included in this study. 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 proximal to 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. On the short arm, one locus contains the genes *Ol-4* and *Ol-6*, which are completely linked to PCR marker GP79L (Fig. 1E). Since the origin of *Ol-6* is unknown, one cannot exclude the possibility that *Ol-4* and *Ol-6* are identical. However, they may also be allelic or linked homologues in a gene cluster, similar to the presumed relationship between *Mi-1* and *Mi-9* in the same chromosomal region. *Mi-9* 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). The region in which one QTL (*Ol-qtl1*; Bai et al. 2003) is most likely located overlaps with *Ol-1* and *Ol-3* as well as with *Ol-5* (Fig. 1E). 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 has been mapped at three loci namely 'MilkyWay', 'Aurora', and 'Orion' (Haanstra et al. 1999a; Yuan et al. 2002). These *Cf* genes belong to a large gene family of *Hcr9s*, which 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 multi-gene families. So, this type of organization is common for resistance loci. It is 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 with considerable allelic variation, like the *L* locus in flax.

Interestingly, the closely linked *Ol* genes on tomato chromosome 6 have similar race specificities and all confer resistance via HR. The *Ol* genes on the long arm (*Ol-1*, *Ol-3*, and *Ol-5*, originating from *L. hirsutum*) were effective to all six *O. neolyopersici* isolates tested, whereas *Ol-4* and *Ol-6* on the short arm showed identical race specificity. The difference in the efficiency in HR between NIL *Ol-4* and *Ol-6* (Table 4) and the different types of hypersensitivity (Fig. 2) may be due to a difference between the two genes (*Ol-4* and *Ol-6*) or to other genes on the introgressed fragment, as NIL *Ol-4* contains a larger introgressed chromosomal segment from *L. peruvianum* (covering both the long arm and the short arm of chromosome 6) than NIL *Ol-6* in which the donor chromosome segment is very small and confined to the short arm. Frequently, homologous genes of the same complex gene cluster confer resistance to different pathotypes of the same pathogen species (Hulbert et al. 2001; Michelmore and Meyers 1998). These homologous genes may have evolved from common ancestors by local gene duplications followed by structural and functional diversification (Gebhardt and Valkonen 2001). Cloning of the *Ol* genes is in progress, and this will provide insight into their structure and organization.

The data presented in this study indicate that the resistance conferred by some *Ol* genes is race-specific and suggest the existence of different *O. neolyopersici* races, which is in agreement with other studies (Kashimoto et al. 2003; Lebeda and Mieslerová 2000). To our knowledge, this is the first study on the interaction of tomato and *O. neolyopersici* using well-defined plant material containing different *Ol* genes in a simi-

Fig. 2. Haustoria, necrotic cells, and papillae in the interaction of tomato and *Oidium neolyopersici* (with isolate On-Ne). **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.

lar 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 NIL will be tested in more locations.

The resistance to *O. neolycopersici* conferred by the dominant *Ol* genes was posthaustorial and associated with HR. HR was restricted to single cells, except for NIL *Ol-4*, in which 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 (Mieslerová 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 had less isogenic *L. esculentum* background. 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 other NIL, not all epidermal cells with haustoria became necrotic and fungal development was generally not stopped but was strongly retarded, as reported by Huang and associates (1998) and Mieslerová and associates (2004). Race-specific resistance is often associated with HR (Dangl and Jones 2001; Lindhout 2002), which is now also proven for the dominant monogenic resistance genes *Ol-4* and *Ol-6*. Since resistance conferred by *Ol-1*, *Ol-3*, and *Ol-5* is also associated with HR, it is likely that *O. neolycopersici* races will appear that can avoid recognition by these *Ol* genes.

Microscopic observation revealed that the recessive *ol-2* gene is associated with papillae formation and not with HR. In F3-*ol-2*, haustorium formation and fungal development were significantly reduced, although fungal penetration was not fully prevented. There are a few other examples of monogenic resistances that are based on non-HR defense mechanisms. The recessive *mlo* gene conferring resistance to *Blumeria graminis* f. sp. *hordei* in barley is involved in papilla formation that prevents fungal haustorium formation and infection (Lyngkjær et al. 2000), while the dominant *Lr34* 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-nonspecific. The resistance conferred by the recessive gene *ol-2* is non-HR and is not race-specific, at least with respect to the *O. neolycopersici* isolates tested so far. Possibly, like the *mlo* gene, *ol-2* governs race-nonspecific 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).

MATERIALS AND METHODS

Plant materials.

The plant materials used in the genetic studies are presented in Table 1. ABL plants 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). By using MM as a recurrent parent, repeated backcrosses were done to develop NIL. Resistant plants were selected by performing disease tests on each backcross generation (BC₁, BC₂, and BC₃). For each dominant *Ol*-gene (*Ol-1*, *Ol-3*, *Ol-4*, *Ol-5*, and *Ol-6*), 96 BC₃S₁ plants from different BC₃ families were subjected to marker assisted selection to select homozygous resistant and susceptible plants by using simple PCR markers linked to the *Ol* genes. The selected BC₃S₁ plants were screened with 12 AFLP primer combinations to characterize the genetic back-

ground. The BC₃S₁ plants (homozygously resistant) were further selfed to produce BC₃S₂ lines that were considered as *Ol* NIL in this study. In addition to the *Ol* NIL of the dominant *Ol* genes, an F₃ line of *L. esculentum* cv. Marmande × *L. esculentum* var. *cerasiforme* carrying homozygously the recessive *ol-2* gene was provided by L. Ricciardi (University of Bari, Italy) and further designated "F3-*ol-2*."

Fungal material.

O. neolycopersici isolates used in this study were collected from infected tomato plants in The Netherlands (*On-Ne*), Hungary (*On-Hu*), the Czech Republic (*On-Cz*), Florida, U.S.A. (*On-Fl*), France (*On-Fr*), and Italy (*On-It*) (Table 3). The *On-Ne* isolate collected in the early 1990s (Lindhout et al. 1994a) has been maintained on MM plants in a growth chamber at 20 °C, 70% relative humidity (RH), and a photoperiod of 16 h.

Disease tests for macroscopic evaluation.

The inoculum preparation and the inoculation were performed as described by Bai and associates (2003). The inoculated plants were grown in a greenhouse at 20–25 °C with 30 to 90% RH. Detailed information of the disease tests for the genetic study and mapping of the *Ol* genes is presented in Table 1. 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 R (no visible fungal sporulation) or S (with fungal sporulation). For incomplete resistance conferred by *Ol-1* or *Ol-5*, inoculated plants were evaluated according to the following DI: 0 = no visible sporulation, 1 = very few fungal spots surrounded by necrosis (weak sporulation), 2 = moderate number of fungal spots (moderate sporulation), and 3 = very high number of fungal spots (heavy sporulation). An average DI was calculated over two times of evaluation (Table 1) for each plant. Plants were considered as R when DI = 1, as S 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.

For the disease tests on the NIL in different geographic locations (Table 3), the experimental set-up was a randomized block design with two blocks. Each block contained five plants of all five *Ol* NIL, F3-*ol-2*, and MM. The inoculated plants were scored according to the DI described above. Four evaluations on fungal development were performed in time with an interval of two days. The first score was on the day when inoculated MM plants started to show symptoms. The DI of each line was calculated by averaging the disease index of all tested plants over the last three time evaluations.

Disease tests for microscopic evaluation in histological study.

For the histological study, a disease test was carried out with the isolate *On-Ne* in a climate chamber at 20–25 °C, with 70–100% RH and a 16-h 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 3×10^5 conidia ml⁻¹, which is about a 15 times higher spore concentration than for a normal inoculation. In order to investigate the resistance mechanism, another experiment was performed with six *Ol* NIL 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 third true leaf (fully expanded) of one-month-old plants was inoculated.

Leaf segments (two per plant) of $1 \times 3 \text{ cm}^2$ were sampled from one of the print-inoculated MM plants of the pilot experiment at 41, 65, and 89 hpi. In the experiment with NIL, two plants per line and per timepoint (41, 65, and 89 hpi) were used for sampling leaf segments (three leaf segments per plant per timepoint). The sampled leaf segments were cleaned and stained with chloral hydrate/trypan blue as described by Huang and associates (1998).

An IU was defined as a germinated spore that produced at least a primary appressorium. A total of 20 IU per leaf segment were scored. The 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 postinoculation, as described above.

Marker analysis.

Total DNA was extracted from leaves of the plants prior to inoculation by using a rapid cetyltrimethylammonium bromide DNA isolation method, as described by Brugmans and associates (2003), and the AFLP fingerprints were generated on a LICOR 4200 DNA sequencer, as described by Bai and associates (2003). BSA was performed on R and S pools that were composed of equal volumes of AFLP pre-amplification products of R and S plants, respectively. AFLP markers were converted into simple PCR markers according to Brugmans and associates (2003). RFLP markers (Tanksley et al. 1992) were converted into simple PCR markers, as described by Bai and associates (2003). Primers for CAPS markers GP79L and 32.5Cla have been published by Kaloshian and associates (1998) and Liharska (1998), respectively. Detailed information about the PCR markers used in this study is presented in Table 2.

Linkage analysis.

The genetic linkage maps were constructed using the software package JOINMAP 3.0 (Kyazma B.V., Wageningen, The Netherlands).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Wageningen University Laboratory of Plant Breeding CAPS markers web-page: www.dpw.wau.nl/pv/CAPStomato

A correction was made to this article on April 7, 2005. The footnote reference letters in Table 1 have been changed.