Theor Appl Genet (2000) 101:661–668

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Mapping strategy for resistance genes against *Cladosporium fulvum* on the short arm of Chromosome 1 of tomato: *Cf-ECP5* near the *Hcr9* Milky Way cluster

Received: 23 August 1999 / Accepted: 12 January 2000

Abstract In the past, numerous *Lycopersicon* accessions have been described that harbor resistance genes to *Cladosporium fulvum* (*Cf* genes). Several *Cf* genes have been isolated, like *Cf-4*, *Cf-4A* and *Cf-9*, which are present on the short arm of Chromosome 1, and *Cf-2* and *Cf-5*, which reside on Chromosome 6. To identify *Cf* genes linked to the *Hcr9* cluster "Milky Way" on the short arm of Chromosome 1, we test-crossed 66 resistant *Lycopersicon* accessions to the near-isogenic line Moneymaker-Cf4, and the F_1s were crossed to the susceptible tomato cultivar Moneymaker. Putative linkage between an unknown *Cf* gene and *Cf-4* was concluded based on small-scale allelic tests from an under-representation of susceptible genotypes in the progenies of 24 plants after inoculation with race 0 of *C. fulvum*. In this way, of the 21 resistant lines tested, 10 harbored a *Cf* gene that was linked to the *Hcr9* Milky Way cluster. Moreover, one of the lines harboring a *Cf* gene closely linked to *Cf-4* specifically recognizes the extracellular protein ECP5 of *C. fulvum* and was designated *Cf-ECP5*. Using a testcross population of 338 plants, we mapped *Cf-ECP5* more accurately at 4 cM proximal to the *Hcr9* Milky Way locus. This report shows that the method of small-scale allelic tests provides a useful tool to rapidly screen for *Cf* genes on the short arm of Chromosome 1. Further analysis of these *Cf* genes will elucidate the complex genetic organization of *Cf* genes on Chromosome 1 of tomato.

Key words Allelism test · *Lycopersicon esculentum* · Avirulence · Hypersensitive response · Genetic variation

Communicated by F. Salamini

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Introduction

There is a long history in breeding for resistance to *Cladosporium fulvum* in tomato (*Lycopersicon esculentum* Mill.). A number of years after the introduction of a specific *Cf* resistance gene into tomato cultivars, the resistance was often rendered ineffective due to changes in the virulence pattern of the fungus. Subsequently, other *Cf* genes from wild *Lycopersicon* species were introduced into tomato cultivars. In this way, several resistance genes have been introduced in tomato cultivars, and intensive studies have been performed to find novel *Cf* genes (Kerr et al. 1971; Kanwar et al. 1980a,b). Since also many different races of *C. fulvum* have been characterized (Lindhout et al. 1989), the interaction between tomato and *C. fulvum* has become one of the best-studied plant-pathogen interactions that fits the gene-for-gene relationship (Joosten and De Wit 1999). The resistance genes *Cf-2* (Dixon et al. 1996), *Cf-4* (Thomas et al. 1997), *Cf-4A* (Takken et al. 1998), *Cf-5* (Dixon et al. 1998) and *Cf-9* (Jones et al. 1994) have been isolated, as well as the avirulence genes *Avr4* (Joosten *et al.* 1994) and *Avr9* (van Kan *et al.* 1991). The isolation of these genes has been an important step in elucidating the mechanism that underlies molecular recognition between resistance gene products and avirulence gene products leading to a hypersensitive response (HR). Numerous breeding lines are still available, harboring yet uncharacterized *Cf* genes (Boukema 1981; Kanwar et al. 1980a,b; Laterrot 1986; Stamova and Yordanov 1978 a,b).

Several resistance genes have been isolated, but an issue that remains to be expounded is the mechanism by which new resistance specificities have developed. Most of the hitherto mapped *Cf* resistance genes are located on

either the short arm of Chromosome 1 (*Cf-4*, *Cf-4A*, *Cf-9*) or on Chromosome 6 (*Cf-2* and *Cf-5*). The short arm of Chromosome 1 contains at least three clusters of *Cf* homologues (designated as *Hcr9* loci). However, only one cluster, designated "Milky Way" is known to harbor functional *Cf* genes (Parniske et al. 1999). Also, in other plant-pathosystems, clusters of resistance genes or resistance gene homologs exist. These clusters can be very different in size and disease specificities (Michelmore and Meyers 1998). For example, in flax several loci conferring resistance to *Melampsora lini* have been identified (Anderson et al. 1997). The *L* locus contains only a single gene, of which 13 alleles are known, while the *M* locus comprises of a tandem array of approximately 15 homologous sequences (Anderson et al. 1997). For the development of tandem array loci, gene duplication events are presumed to have occurred. In tomato, complete *Hcr9* loci might have been duplicated, because the sequences flanking these loci are very similar (Parniske et al. 1999). However, in between these loci unique DNA sequences have been identified, which is not to be expected if these genes originated from large duplication events (Parniske et al. 1999). The birth-and-death model presented by Michelmore and Meyers (1998) proposes that unequal crossing-over event are responsible for these duplication events. A high amount of sequence similarity in the intergenic regions may provoke new unequal crossing-over events. This is consistent with the findings of Parniske et al. (1997) who found that in a *Cf-4*/*Cf-9* heterozygote unequal crossing-overs occurred in the intergenic regions with high sequence similarity rather than within genes, thus preventing homogenization of *Cf* genes. Although this might explain the variation of *Hcr9s* at one locus, it does not explain the presence of multiple *Hcr9* loci. The presence of open reading frames of *Hcr9* genes outside the Milky Way cluster suggests that there may be functional *Cf* genes in these *Hcr9* clusters (Parniske et al. 1999). The isolation of resistance genes from other accessions might better clarify the development of new genes with other specificities in the perspective of the birth-and-death model. In principle, these genes might be present in all *Solanaceae*, since

Table 1 Accessions which have been tested for resistance to *C. fulvum* race 0, for necrotic response to AVR9 using PVX::*Avr9* and for segregation of resistance to *C. fulvum* race 0 in testcross progenies

Number	Accession[a]	Designation of resistance[b]	Race 0[c]	PVX::Avr9[d]	Segregation[e]	Conclusion[f]
1	*L. esc* G1.1145	Cf-1	IR	–	NT	
2	*L. esc* G1.1146	*Cf-2*	R	–	8/24	Unlinked
3	*L. esc* G1.1147	*Cf-3*	R	–	NT	
4	*L. esc* G1.1148	*Cf-4*	R	–	NT	
5	*L. esc* G1.1149	*Cf-5*	R	–	NT	
6	*L. esc* G1.1150	*Cf-?*	R	–	NT	
7	*L. esc* G1.1151	*Cf-?*	R	–	4/24	Unlinked
8	*L. esc* G1.1152	*Cf-?*	R	–	NT	
9	*L. esc* G1.1153	*Cf-?* (*Cf-ECP3*)	R	–	0/24	Linked/allelic
10	*L. esc* G1.1154	Cf-9	R	+	NT	
11	*L. esc* G1.1155	F_4BC_5	R	–	5/24	Unlinked
12	*L. esc* G1.1156	F_4BC_5	R	–	NT	
13	*L. esc* G1.1157	F_3BC_5	R	–	NT	
14	*L. esc* G1.1158	F_3BC_5	R	–	7/24	Unlinked
15	*L. esc* G1.1159	F_3BC_5	R	–	3/24+5/24	Unlinked
16	*L. esc* G1.1160	F_3BC_5	R	–	NT	
17	*L. esc* G1.1161	F_3BC_5 (*Cf-ECP5*)	R	–	1/24+2/339	Linked
18	*L. esc* G1.1162	F_3BC_5	R	–	NT	
19	*L. esc* G1.1163	F_3BC_5	R	–	2/24	Linked
20	*L. esc* G1.1164	F_3BC_5	R	–	8/24	Unlinked
21	*L. esc* G1.1165	F_3BC_5	R	–	NT	
22	*L. esc* G1.1653	Cf-11	R	–	NT	
23	*L. esc* G1.1656	*Cf-6*	R	–	9/24	Unlinked
24	*L. esc* 881271	*Cf-8*	R	–	NT	
25	*L. esc* LA2443	Cf-1	IR	NT	NT	
26	*L. esc* LA2446	*Cf-4*	R	–	NT	
27	*L. esc* LA3045	*Cf-4*	R	–	NT	
28	*L. esc* LA3047	*Cf-9*	R	NT	NT	
29	*L. esc* LA3048	*Cf-?*	R	–	NT	
30	*L. esc* LA3049	*Cf-?*	R	–	0/18+5/181	Linked
31	*L. esc* LA3050	*Cf-?*	R	–	NT	
32	*L. esc* LA3051	*Cf-?*	R	–	NT	
33	*L. esc* LA3266	*Cf-?*	R	–	NT	
34	*L. esc* LA3267	*Cf-?*	R	–	NT	
35	*L. esc* LA3271	*Cf-?*(*Cf-ECP3*)	R	–	NT	
36	*L. esc* LA3272	Cf-?	R	–	NT	
37	*L. esc* LA3431	*Cf-?*	R	–	NT	
38	*L.pim* CGN14354	*Cf-?*	R	–	1/24	Linked
39	*L. esc* CGN15397	*Cf-4*	R	–	NT	

all solanaceous species tested harbor *Cf* homologues (Kooman-Gersmann et al. 1996). Moreover, Laugé et al. (1999) identified isolated extracellular proteins (ECPs) of *C. fulvum* in *Nicotiana paniculata*, indicating the presence of functional *Cf* genes in these species. However, since *C. fulvum* is only pathogenic on tomato, only in *Lycopersicon* species can these *Cf* genes have a function in the resistance to this fungus. Alternatively, avirulence proteins (AVRs) and ECPs may be conserved in other plant pathogenic fungi.

Recently, we have mapped a resistance gene, *Cf-ECP2*, conferring resistance to *C. fulvum* through recognition of the extracellular protein ECP2. The mapping of *Cf-ECP2* on Chromosome 1, between the Milky Way and Southern Cross clusters (Haanstra *et al.* 1999), presented additional evidence for the presence of functional *Cf* genes on Chromosome 1 that are not part of the Milky Way cluster. In order to study the genetic variation and organization of *Cf* genes on the short arm of tomato Chromosome 1 we describe here the results from a screening of 66 *Lycopersicon* accessions for *Cf* resistance genes on the short arm of Chromosome 1. The variation in map position and specificity of the *Cf* genes is discussed with respect to the development of new resistance specificities.

Materials and methods

Plant material and disease tests

Sixty-six *Lycopersicon* accessions with reported *Cf* resistance were collected from various gene banks (Table 1). Seeds, kindly donated by the Center for Genetic Resources Netherlands, Wageningen, The Netherlands (CGN) and the Center for Plant Breeding and Reproduction Research, Wageningen, The Netherlands (CPRO), originate from the pioneer work of I.W. Boukema and seeds, kindly donated by the Plant Genetic Resources Canada, Ottawa, Canada (PGRC), result from the pioneer work of Dr. E.A. Kerr. Seeds of several *C. fulvum*-resistant accessions, as reported in the SOLGENES Internet database, were provided by the C.M. Rick Tomato Genetic Resources Center, UC Davis, USA (TGRC). To confirm *Cf* resistance, we tested accessions at the two-leaf stage for resistance to *Cladosporium fulvum* race 0, a race that contains all known avirulence genes, by inoculation with a sus-

Table 1 (continued)

Number	Accession[a]	Designation of resistance[b]	Race 0[c]	PVX::Avr9[d]	Segregation[e]	Conclusion[f]
40	*L.pim* CGN14353	*Cf-?*	R	–	NT	
41	*L.pim* CGN15529	*Cf-?*	R	–	NT	
42	*L.pim* CGN15808	*Cf-ECP2*	R	+	NT	
43	*L.pim* CGN15814	*Cf-9*?	R	–	0/24	Linked/allelic
44	*L.min* CGN15815	Cf-?	R	–	NT	
45	*L. esc* CGN15839	*Cf-6*	R	–	NT	
46	*L. esc* CGN15840	*Cf-15*	R	–	7/18	Unlinked
47	*L. esc* CN0078	Cf-1,Cf-2,Cf-3,Cf-4	NT	–	NT	
48	*L. esc* CN0335	*Cf-1,Cf-2,Cf-4*	NT	–	NT	
49	*L. esc* CN0354	*Cf-2,Cf-4*	NT	–	NT	
50	*L. esc* CN0355	*Cf-1,Cf-2,Cf-4*	NT	–	NT	
51	*L. esc* CN0356	*Cf-1,Cf-2,Cf-3,Cf-4*	NT	–	NT	
52	*L. esc* CN0697	*Cf-2,Cf-4*	NT	–	NT	
53	*L. esc* CN0698	*Cf-1,Cf-2,Cf-3,Cf-4*	NT	–	NT	
54	*L. esc* CN1711	*Cf-23* (*Cf-ECP2*)	NT	–	NT	
55	*L. esc* CN2002	Cf-8	NT	–	NT	
56	*L. esc* CN2418	*Cf-12*	NT	–	NT	
57	*L. esc* CN6762	*Cf-18* (*Cf-ECP2*)	NT	–	0/24	Linked/allelic
58	*L. esc* CN6763	*Cf-20* (*Cf-ECP2*)	NT	–	2/24	Linked
59	*L. esc* CN6764	*Cf-21*	NT	–	7/24	Unlinked
60	*L. esc* CN6765	*Cf-22*	NT	–	2/24	Linked
61	*L. esc* CN6766	*Cf-13*	NT	–	NT	
62	*L. esc* CN6767	*Cf-14*	NT	–	2/24	Linked
63	*L. esc* CN6768	Cf-16	NT	–	7/24	Unlinked
64	*L. esc* CN6770	*Cf-24* (*Cf-ECP2*)	NT	–	NT	
65	*L. esc* CN7494	*Cf-17*	NT	–	6/24	Unlinked
66	*L. esc* IVT771891	*Cf-?*	NT	–	NT	

[a] *L.esc*, *Lycopersicon esculentum*; *L.pim*, *L. pimpinellifolium*; *L.min*, *L. minutum*. Lines were obtained from: CPRO, Center for Plant Breeding and Reproduction Research, Wageningen, The Netherlands (lines 1–24, 66); TGRC, Tomato Genetic Resource Center, UC Davis, USA (lines 25–37); CGN, Center for Genetic Resources Netherlands (lines 38–46); and PGRC, Plant Genetic Resources Canada, Ottawa, Canada (lines 47–65)

[b] *Cf* genes as they have been previously designated in literature, *Cf-?*, uncharacterized *Cf* resistance; F_4BC_5 and F_3BC_5 indicate that the resistance in those lines is obtained from resistant wild *Lycopersicon* species after backcrossing to the susceptible tomato cultivar Moneymaker, followed by selfing and selection for homozygous resistant plants

[c] IR, incomplete resistance to *C. fulvum* race 0; R, resistance to *C. fulvum* race 0; NT, not tested with *C. fulvum* race 0

[d] +, necrosis upon infection by PVX::*Avr9*; -, mosaic upon infection by PVX::*Avr9*; NT, not tested with PVX::*Avr9*

[e] The number in front of the dash (/) indicates the number of susceptible plants per testcross population upon inoculation with *C. fulvum* race 0. The number behind the dash (/) indicates the number of plants tested per progeny. NT, Not tested

[f] Linked and allelic indicates that the resistance gene in those lines is linked, respectively allelic, to *Cf-4*. Unlinked indicates that the resistance in those lines is not linked to *Cf-4*

Table 2 Primer sequences, annealing temperatures, lengths of amplified PCR products and restriction enzymes revealing CAPS markers between *L. esculentum* cv. Moneymaker (MM), Moneymaker-Cf4 (MM-Cf4) and CfECP5

Locus	Primer sequence	Annealing temperature (°C)	PCR product size (bp)[a]	Enzymes detecting *polymorphism*	Total number of enzymes tested
CT233	See Bonnema *et al*. (1997)	54	1650	–	43/43
CT2	f: AAG CCT CTA ATC AAG AAA ATG G r:TTC AGT GCA ATA ATA ATG AGG G	50	490	*Mse*I[1,2]	20/11
TG301	See Bonnema *et al*. (1997)	57	800	*Alu*I[1,2],*Rsa*I[1,2]	41/41
CP46	See Bonnema *et al*. (1997)	50	1000	*Dde*I[3], *Hinf*I[1,2], *Mbo*II[2,3]	14/7
FT33	f: AGA AGG ATA AAG CTC AAC ATC GG r: AAG GAA CAT CTG TGG TTC GC	58	1100	–	13/13
TG236	See Bonnema *et al*. (1997)	57	1000	*Ssp*I[2,3]	32/26
CT268	f: ATG AAA ATG CTC AAA TGT TGT TG r: CTT GGA TCT TCT GGA TTC TAC TAC C	57	300	–	4/4
CT116	See Bonnema *et al*. (1997)	60	1700	*Hha*I[2,3],*Mse*I[2,3]	24/24
TG67	See Bonnema *et al*. (1997)	59	1000	–	42/28
TG184	See Bonnema *et al*. (1997)	59	1155	–	17/17
CT209	See Bonnema *et al*. (1997)	60	600	–	33/33
TG51	See Bonnema *et al*. (1997)	58	1500	*Hinf*I[3]	42/39
Cf-4	f3: TTT CAT GCT ATA TGT CTT TCT C r3: AAT TGG TCC TTC AAG ATG GTT A	54	1000	*Eco*RI[1,2,3]	1/1

[a] 1 = Polymorphism between MM and MM-Cf4, [2]= polymorphism between CfECP5 and MM-Cf4 and [3]= polymorphism between MM and CfECP5

[b] The number before the dash (/) indicates the number of enzymes tested for MM and CfECP5 only and the number behind the dash (/) indicates the number of enzymes tested on all three genotypes

pension of spores, stage as described previously (Haanstra *et al*. 1999). Accessions were crossed to the near-isogenic line Moneymaker-Cf4 (MM-Cf4). Subsequently, the F_1 was crossed to the susceptible tomato cultivar Moneymaker (MM). These crosses are referred to as 'testcrosses', and their progenies as 'testcross progenies'. The *L. esculentum* line G1.1161 was found to show a specific HR upon exposure to the fungal extracellular protein ECP5 (Laugé et al. 1999) and was designated "CfECP5". F_3 lines of 231 F_2 plants, derived from the cross MM x CfECP5 were obtained for segregation tests of *Cf-ECP5* resistance.

Potato virus X (PVX) assays

All *Lycopersicon* accessions were tested for the presence of *Cf-4* or *Cf-9* by inoculation of plants at the two-leaf stage with recombinant PVX expressing the *Avr4* gene (PVX::*Avr4*; Joosten et al. 1997) or the *Avr9* gene (PVX::*Avr9*; Hammond-Kosack et al. 1995). Fourteen days after inoculation, plants were scored visually in two classes: necrotic (HR, indicating presence of *Cf-4* or *Cf-9*) or mosaic (no HR, indicating absence of *Cf-4* or *Cf-9*). From each of the 231 F_3 lines of the cross MM × CfECP5, 20–40 plants were tested for the segregation of *Cf-ECP5* by the inoculation of seedlings with recombinant PVX carrying the *Ecp5* gene (Laugé et al. 1999), similar to the other PVX inoculation assays.

Linkage analysis between *Cf* genes

To study the linkage between an unidentified *Cf* gene and *Cf-4*, the testcross progenies were inoculated with *C. fulvum* race 0 as described above. The genetic distance between these *Cf* genes was estimated from the frequency of susceptible recombinants. After the evaluation of the disease tests with race 0, all diseased leaves of the testcross progeny MM × (MM-Cf4 × CfECP5) were removed, and the plants were grown for 10–14 days under conditions with a low relative humidity to prevent further growth of *C. fulvum.* Subsequently, this testcross population was tested for resistance to race 2.4.5 as described for race 0. Race 2.4.5 is pathogenic on plants harboring any combination of the resistance genes *Cf-2, Cf-4* or *Cf-5*. Also, cuttings of 2-month-old plants of this testcross population were assayed with PVX::*Avr4* as described above.

DNA isolation, CAPS analysis and genetic linkage analysis

DNA was isolated according to the protocol described by Van der Beek *et al*. (1992). DNA of the parents CfECP5, MM and MM-Cf4 was used to screen for cleaved amplified polymorphic sequence (CAPS) markers on the short arm of Chromosome 1 as described by Haanstra *et al*. (1999a) (Table 2). The testcross progeny MM × (MM-Cf4 × CfECP5) and the F_2 progeny of 233 plants of MM × CfECP5 were tested for segregation of these CAPS markers. An integrated genetic map was calculated using the computer package JoinMap 2.0 (Stam and Van Ooijen 1996).

Results

Cf resistance in parents

In the past, several researchers have searched for novel sources of *Cf* resistance. They developed the breeding material that we obtained from gene banks and used for the research described here. Most *Lycopersicon* accessions obtained were tested for resistance to *C. fulvum* race 0. All tested accessions were confirmed to be resistant (Table 1). The accessions *L. esculentum* G1.1145 and *L. esculentum* LA2443, harboring the resistance gene *Cf-1*, showed less sporulation than the MM control plants, and were considered to be incomplete resistant.

Presence of *Cf-4* and *Cf-9* in the *Lycopersicon* accessions

The genes *Cf-4* and *Cf-9* have been frequently used in breeding programs, and numerous tomato lines harboring these genes are available. In addition, *Cf-4* and *Cf-9* have been mapped on Chromosome 1 and have been isolated (Thomas *et al.* 1997; Jones *et al.* 1994). In order to avoid confusion of putative novel *Cf* genes with *Cf-4* and *Cf-9* we tested the *Lycopersicon* accessions for the presence of *Cf-4* and *Cf-9* using PVX::*Avr4* and PVX::*Avr9* assays, respectively. Upon inoculation with PVX::*Avr4*, 17 genotypes exhibited necrosis (Haanstra and Lindhout, 2000). Upon inoculation with PVX::*Avr9*, only MM-Cf9 (*L. esculentum* G1.1154) and *L. pimpinellifolium* PI126947 (CGN15808) showed necrosis (Table 1). Consequently, genotypes responding with necrosis to either of the two recombinant PVX strains were not tested for linkage to *Cf-4* or *Cf-9*.

Test for linkage of putative *Cf* genes to the *Cf-4*/*Cf-9* cluster

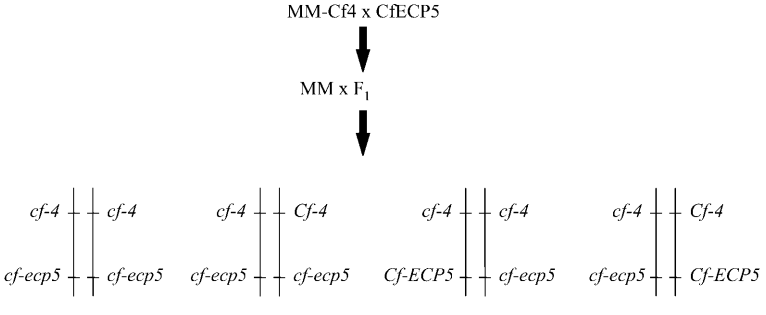
A number of randomly chosen resistant accessions which did show necrosis upon inoculation with PVX::*Avr4* or PVX::*Avr9* were tested for carrying a *Cf* gene allelic to *Cf-4* (Table 1). In most trials, 24 plants per testcross population were tested for segregation of resistance to *C. fulvum* race 0. If 2 or fewer showed disease symptoms, we concluded that the uncharacterized *Cf* gene was probably linked to the *Hcr9* Milky Way cluster ($P<0.06$). From the 21 accessions tested, we identified 10 accessions with a *Cf* gene that was putatively linked to the *Hcr9* Milky Way cluster (Table 1). To confirm linkage, we tested another 181 plants of the testcross population of *L. esculentum* LA3049, a randomly chosen testcross population that did not contain even 1 susceptible plant out of the 18 initially tested. Only 5 were susceptible to *C. fulvum* race 0 (Table 1). Assuming the presence of a single *Cf* gene in *L. esculentum* LA3049, this gene is expected to be at a distance of 5.5 cM from the *Hcr9* Milky Way cluster. It is not known from which accession the resistance in LA3049 originated, but the *Cf* resistance is present in a Moneymaker background (R. Chetelat, personal communication).

Test for linkage between *Cf-ECP5* and *Cf-4*

The *L. esculentum* accession G1.1161 (CfECP5) was an F_3BC_5 of MM with *L. pimpinellifolium* CGN15529 (I.W. Boukema, personal communication) and showed a specific HR to the extracellular protein ECP5 from *C. fulvum* (Laugé *et al.* 1999). This *Cf* gene has been designated *Cf-ECP5*. Initially, a testcross of CfECP5 with MM-Cf4 only showed 1 susceptible plant out of 24 tested (Table 1). A larger population of 338 offspring of the same testcross was evaluated for segregation of resistance to *C. fulvum* race 0. Only 2 plants appeared to be susceptible, confirming the close linkage of *Cf-ECP5* with *Cf-4*. In addition, all offspring were inoculated with race 2.4.5 to identify plants harboring only *Cf-4*, as this race is able to overcome *Cf-4*, but not *Cf-ECP5*. Finally, PVX::*Avr4* assays were used to discriminate plants harboring only *Cf-ECP5* from the plants harboring both *Cf-4* and *Cf-ECP5* (Fig. 1). Segregation into genotypes *cf-4cf-4*/*cf-ecp5cf-ecp5*, *Cf-4cf-4*/*cf-ecp5cf-ecp5*, *cf-4cf-4*/*Cf-ECP5cf-ecp5* and *Cf-4cf-4*/*Cf-ECP5cf-ecp5* was 2:158:167:11. This gives a distance of 3.8 ± 1.0 cM between *Cf-4* and *Cf-ECP5*.

CAPS analysis and mapping of *Cf-ECP5*

To further identify the map position of *Cf-ECP5*, we screened the parent genotypes MM, MM-Cf4 and CfECP5 for CAPS markers on the short arm of Chromosome 1 (Table 2). Out of the 325 primer- restriction enzyme combinations tested, we detected ten polymorphisms, representing 6 of the 12 marker loci tested (Table 2). Furthermore, to confirm the results of the dis-



Response to *C. fulvum* and PVX inoculations:

C. fulvum race 0	S	R	R	R
C. fulvum race 2.4.5	S	S	R	R
PVX::*Avr4*	M	N	M	N
# observed	2	158	167	11

Fig. 1 Construction and analysis of a testcross population of CfECP5. *R* Resistance, *S* susceptibility. *M* indicates mild mosaic symptoms and *N* systemic necrosis upon inoculation with PVX::*Avr4*. The number of plants per genotype class are indicated by *# observed*

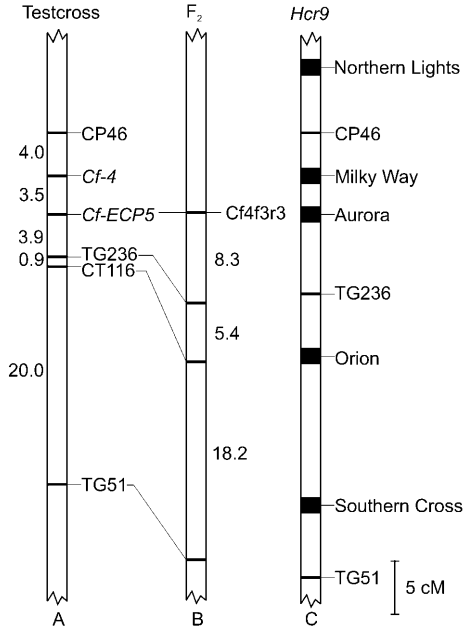


Fig. 2 Genetic maps of the short arm of Chromosome 1 around *Cf-ECP5*. *Map A* is based on the progeny of the cross MM × (MM-Cf4 × CfECP5), *map B* on the F_2 population of the cross MM × CfECP5. Map distances (in cM) have been calculated using the software package JoinMap 2.0 (Stam and Van Ooijen 1996). *Map C* shows the presence of all *Hcr9* loci so far identified on the short arm of Chromosome 1. The location of Northern Lights and Southern Cross were retrieved from Parniske et al. (1999), the location of Orion from Haanstra et al. (1999) and the locations of Milky Way and Aurora resulted from the present study. Distances between markers are obtained from Bonnema et al. (1997).

ease tests on the testcross population, the we digested the polymerase chain reaction (PCR) product of Cf4f3r3 with *Eco*RI (Table 2). This CAPS gives a *Cf-4* specific band of 842 bp that is absent in MM and MM-Cf9 (F. Takken, personal communication) and is therefore diagnostic for the presence of *Cf-4* in the testcross progeny. However, CfECP5 gave a slightly larger band of approximately 870 bp. The *Cf-4*- and CfECP5-specific bands could not be separated on an agarose gel and were therefore not useful for screening of the testcross population. The CfECP5-specific band was mapped using the F_2 population of MM × CfECP5. Of the testcross population, 295 plants were screened with the CAPS marker CP46-*Hinf*I, and 50 plants with TG236-*Ssp*I, CT116-*Hha*I and TG51-*Hinf*I. The F_2 population of 233 plants derived from the cross MM × CfECP5 was evaluated for the segregation of the CAPS markers Cf4f3r3-*Eco*RI, TG236-*Ssp*I, CT116-*Hha*I and TG51-*Hinf*I. The genotype of the *Cf-ECP5* locus was determined by testing the F_3 lines from the F_2 population of MM × CfECP5 for the segregation of *Cf-ECP5* using PVX::*Ecp5* inoculation assays. From the 231 lines tested, 166 lines showed clear segregation for presence or absence of necrosis. It was often impossible to determine whether the non-necrotic plants exhibited mild mosaic symptoms (indicating a successful PVX infection and consequently absence of *Cf-ECP5*) or whether they escaped entirely from infection by PVX. Therefore, these lines were scored as dominant for the *Cf-ECP5* locus. In the remaining 65 F_3 lines, no necrosis was observed in any plant. Consequently, the corresponding F_2 plants were classified as homozygous *cf-ecp5*/*cf-ecp5*. Maps of the testcross population and the F_2 were constructed (Fig. 2). The dominant marker Cf4f3r3-*Eco*RI cosegregated with *Cf-ECP5* in the F_2 population. *Cf-ECP5* mapped proximal to the *Hcr9* Milky Way cluster, but distal to CT116, which is cosegregating with *Cf-ECP2* (Haanstra *et al.* 1999).

Discussion

Cf resistance genes on the short arm of Chromosome 1

In the present study we tested 21 lines for the presence of *Cf* genes that are linked to the *Hcr9* Milky Way cluster on the short arm of Chromosome 1 of tomato. We identified 10 accessions with just such putative *Cf* genes, 4 of which showed a specific response to three different extracellular proteins (ECPs) of *Cladosporium fulvum*: 2 accessions responded to ECP2, one to ECP3 and 1 to ECP5 (Table 1). For the other 6 accessions the interaction with specific avirulence factors is still obscure, but as they did not respond to the known avirulence genes, at least one additional resistance gene is present in these lines. In conclusion, at least four unknown *Cf* geneswere characterized by map position and specificity. Using molecular marker analyses, we were able to show that *Cf-ECP2* and *Cf-ECP5* are closely linked to the *Hcr9* Milky Way cluster. Most likely, the remaining 7 other accessions also harbor *Cf* genes near or at the *Hcr9* Milky Way cluster.

Most *Cf* genes investigated in this study originate from *L. pimpinellifolium*. It is likely that even more *Cf* genes are present in the other *Lycopersicon* species, as these species show more genetic variation than *L. pimpinellifolium* (Miller and Tanksley 1990). In the past, breeders and researchers preferred to study *L. pimpinellifolium* as this species is the most closely related to *L. esculentum* and hence is the easiest exploitable species from which to transfer *Cf* genes to the cultivated tomato. An example of a *Cf* gene in another wild species is the *Cf-4* gene, which probably originates from *L. hirsutum* (Kerr and Bailey 1964; Thomas et al. 1997). *Cf* genes originating from *L. pennellii*, (Stamova et al. 1985) *L. peruvianum* (Kerr and Bailey 1964), *L. chilense* (Stamova and Yordanov 1978a) and *L. cheesmanii* (Stamova and Yordanov 1978b) have also been reported. In addition, other solanaceous species have been reported to harbor *Cf* homologes (Kooman-Gersmann *et al.* 1996) or can specifically recognize ECPs (Laugé *et al.* 1999). The characterization of *Cf* gene clusters in other tomato and solanaceous species will be of great significance to extend our knowledge on the variation in specificity and structure of *Cf* resistance genes.

Mapping of *Cf-ECP5*

One of the accessions (CfECP5) harbored a *Cf* gene on the short arm of Chromosome 1 that responded to exposure to the *C. fulvum* extracellular protein ECP5, which was isolated by Laugé *et al*. (1999). This gene that was designated *Cf-ECP5* was mapped using different strategies. Firstly, a large testcross population was evaluated for resistance to the *C. fulvum* races 0 and 2.4.5 and for reaction to PVX::*Avr4* to determine the segregation and hence the linkage of *Cf-4* and *Cf-ECP5*. Secondly, an analysis with Chromosome 1-specific CAPS markers was performed to more accurately determine the genetic distance between these *Cf* genes and marker loci on the short arm of Chromosome 1. Thirdly, F_3 lines of a cross between MM and CfECP5 were inoculated with PVX::*Ecp5* to determine the genotype of the *Cf-ECP5* locus in the F_2 plants. This assay also provided additional information on the distance between *Cf-ECP5* and several marker loci on the short arm of Chromosome 1. There were differences between the map that was based upon the testcross progeny and the map based on the F_2 population. Whereas the distance between CT116 and TG236 was 1 cM in the tescross progeny, this distance was 6 cM in the F_2 population. We have previously mapped *Cf-ECP2* using a similar F_2 population. Like *Cf-ECP5*, *Cf-ECP2* also originates from a wild *L. pimpinellifolium* species. In the latter F_2 population, the distance between CT116 and TG236 was 4.5 cM (Haanstra et al. 1999). This closer distance resembles that between CT116 and TG236 observed in the F_2 mapping population rather than that observed in the testcross population of CfECP5. However, the distance between CT116 and TG51 in the F_2 and tescross population with *Cf-ECP5* was 18.1 and 20.0 cM, respectively, which is more similar. It has been previously shown that recombination frequencies are decreased around the TG236 locus in a cross between *L. hirsutum* (the supposed donor of *Cf-4*) and *L. esculentum* relative to recombination frequencies in a cross between *L. peruvianum* and *L. esculentum*. The introgression segment of *Cf-4* in MM extends over TG236 but not over TG51 (Parniske et al. 1999). *L. pimpinellifolium* (the donor of *Cf-ECP5*) resembles *L. esculentum*, and therefore shows normal recombination in crosses with *L. esculentum* and decreased recombination frequencies around TG236 in crosses with *L. hirsutum*. The decreased recombination around TG236 in crosses with *L. hirsutum* may explain the variation in recombination differences between both mapping populations as well as between different markers within one mapping population.

In conclusion, we have mapped *Cf-ECP5* on the short arm of Chromosome 1, between the *Hcr9* Milky Way cluster and CT116. The CAPS marker Cf4f3r3-*Eco*RI, which is based upon sequence homology to *Cf-4*, cosegregated with *Cf-ECP5*, indicating the presence of an *Hcr9* at this locus. Previously, we have mapped *Cf-ECP2*, which is at an *Hcr9* locus that is closely linked to CT116 (Haanstra *et al.* 1999). This locus has been designated "Orion". Since no *Hcr9* clusters have been mapped between Milky Way and Orion before, we have designated the *Hcr9* locus containing *Cf-ECP5* "Aurora".

Acknowledgements We acknowledge I.W. Boukema (Center for Genetic Resources Netherlands), G. Pet (Center for Plant Breeding and Reproduction Research, Wageningen, The Netherlands), R.T. Chetelat (C.M. Rick Tomato Genetic Resources Center, UC Davis, USA) and S. Wallace (Plant Genetic Resources Canada, Ottawa, Canada) for providing seeds of the tomato accessions used in this research. We thank I.W. Boukema for helpful discussions on the breeding history of the accessions. J. Haanstra was financially supported by the Ministry of Economic Affairs, the Ministry of Education, Culture and Science, the Ministry of Agriculture, Nature Management and Fishery in the framework of an industrial relevant research programme of the Netherlands Association of Biotechnology Centres in the Netherlands (ABON). R. Laugé was supported by the European Community-Human Capital and Mobility program ERBCHRXCT930244. M.H.A.J. Joosten was supported by Zeneca-Mogen (Leiden, The Netherlands).

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