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# The *Cf-ECP2* gene is linked to, but not part of, the *Cf-4/Cf-9* cluster on the short arm of chromosome 1 in tomato

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**Abstract** A gene has been identified in tomato, which confers resistance to *Cladosporium fulvum* through recognition of the pathogenicity factor ECP2. Segregation analysis of $F_2$ and $F_3$ populations showed monogenic dominant inheritance, as for previously reported *Cf* resistances. The gene has been designated *Cf-ECP2*. Using several mapping populations, *Cf-ECP2* was accurately mapped on chromosome 1, 7.7 cM proximal to TG236 and 6.0 cM distal to TG184. Although *Cf-ECP2* is linked to *Cf-4*, it is not located in the *Hcr9* cluster "Milky Way". Therefore, *Cf-ECP2* is the first functional *Cf* homologue on chromosome 1 that does not belong to this *Hcr9* cluster. No recombination events between *Cf-ECP2* and CT116 have been observed in three populations tested, representing 282 individuals. The low value for the physical distance per cM around CT116 reported previously and the high probability that *Cf-ECP2* is also a member of a *Hcr9* cluster will facilitate cloning of the locus.

**Key words** *Lycopersicon esculentum* · *Cladosporium fulvum* · Pathogenicity · CAPS markers · *Hcr9*

## Introduction

The relationship between the cultivated tomato (*Lycopersicon esculentum*) and *Cladosporium fulvum* Cke., the causal agent of leaf mould, is one of the best studied plant pathosystems (Van den Ackerveken and De Wit 1994). Since the 1930s many genes for resistance to *C. fulvum* have been identified in wild tomato species and subsequently introgressed into the cultivated tomato (Stevens and Rick 1988). However, soon after the introduction of resistant cultivars, new races of *C. fulvum* have appeared, that rendered these resistances ineffective (Bailey and Kerr 1964; Laterrot et al. 1985; Laterrot 1986). This phenomenon of non-durable resistance has been observed in many plant-pathogen relationships. The gene-for-gene hypothesis (Flor 1942) explained the non-durability of plant-pathogen interactions. The tomato-*C. fulvum* interaction has proved to be an ideal model system for the study of the gene-for-gene interaction. The plant resistance genes *Cf-4* (Thomas et al. 1998) and *Cf-9* (Jones et al. 1994) have now been isolated, together with the corresponding fungal avirulence genes *Avr4* (Joosten et al. 1994) and *Avr9* (Van den Ackerveken et al. 1992), respectively. Fungal races that were able to circumvent the *Cf-4* resistance were found to carry mutant *Avr4* alleles (Joosten et al. 1994) that differed by only a single base-pair from the wild-type *Avr4* allele, while fungal races which were able to colonize plants carrying the *Cf-9* resistance gene lacked the *Avr9* gene completely (Van Kan et al. 1991).

Another *C. fulvum* gene, *Ecp2*, that plays an important role in the pathogenicity has also been isolated (Van den Ackerveken et al. 1993; Laugé et al. 1997). Gene replacement studies showed that strains lacking *Ecp2* colonized the leaf poorly and produced only few conidia (Laugé et al. 1997). It is to be expected that *Cf* genes that confer resistance following the recognition of pathogenicity factors could give rise to a more durable resistance, because an isolate with an alteration in the corresponding *Avr* gene may lose its pathogenicity. In a survey of 21 *Lycopersicon* accessions, four lines were identified that showed a specific hypersensitivity reaction (HR) upon inoculation with a recombinant potato virus X derivative that expresses *Ecp2* (Laugé et al. 1998). These lines all originated from one *L. pimpinellifolium*

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accession (PI126947), which also showed a specific necrosis reaction to ECP2 (Laugé et al. 1998). Boukema (1980) provided evidence that this *L. pimpinellifolium* accession contained a resistance gene that is allelic to *Cf-9*. Most *Cf* genes mapped so far are closely linked or allelic and map either on chromosome 1 or chromosome 6 (Dickinson et al. 1993; Balint-Kurti et al. 1994). Molecular analyses have demonstrated the presence of three clusters that contain *Cf* homologues on the short arm of chromosome 1, hereafter referred to as *Hcr9* clusters (Parniske et al. 1998). The functional *Cf* genes on chromosome 1 that have been cloned so far, *Cf-4, Cf-4A* and *Cf-9* (Jones et al. 1994; Takken et al. 1998; Thomas et al. 1998), all reside in one of these *Hcr9* clusters, designated the Milky Way cluster (Parniske et al. 1998). It was hypothesized that *Cf-ECP2* could be located in one of the three clusters on the short arm of chromosome 1. This report describes the accurate mapping of *Cf-ECP2* on the short arm of chromosome 1 of tomato between two of the three previously reported *Hcr9* clusters, Milky Way and Southern Cross.

## Material and methods

### Plant material

From a collection of *C. fulvum*-resistant genotypes, the four breeding lines Ontario 7518 (Cf18), Ontario 7520 (Cf20), Ontario 7523 (Cf23) and Ontario 7819 (Cf24) (Kanwar et al. 1980), as well as their wild ancestor *L. pimpinellifolium* PI126947, showed systemic HR upon infection with a potato virus X (PVX) strain that expresses the *Ecp2* gene (PVX::*Ecp2*; Laugé et al. 1998). Three of these lines, Cf18, Cf20, and Cf24, were crossed to the near-isogenic line Moneymaker-Cf4 (MM-Cf4). Subsequently, the $F_1$ plants were crossed to the susceptible tomato cultivar Moneymaker (MM). The progenies of these testcrosses were designated TCf18, TCf20 and TCf24, respectively.

An $F_2$ population of 106 plants obtained from the cross MM × Ontario 7518 ($F_2$Cf18) was selfed, yielding an $F_3$ that was designated $F_3$Cf18.

### *C. fulvum* resistance test

A strain of *C. fulvum* race 0 was obtained from the IPO-DLO (Wageningen, The Netherlands). The fungus was grown on potato dextrose agar (Duchefa, Haarlem, The Netherlands) at 22° C and sporulated abundantly after about 14 days. Twenty-four plants from each of the populations TCf18 and TCf20, as well as MM and MM-Cf4 control plants, were inoculated and scored as described by Lindhout et al. (1989) with some minor modifications. A relative humidity of 100% was maintained for 24 h by using a humidifier. Plants at the two-true-leaf stage were inoculated twice, 2 days apart.

### PVX::*Avr4*, PVX::*Avr9* and PVX::*Ecp2* assays

Plants of the breeding lines Cf18, Cf20, Cf23, Cf24 and their ancestor *L. pimpinellifolium* PI126947 were inoculated at the two-leaf stage with a PVX deriviative expressing the *Avr4* gene (PVX::*Avr4*; Joosten et al. 1997) and with PVX::*Avr9* (Hammond-Kosack et al. 1995). Two weeks after inoculation the plants were scored visually and assigned to one of two classes: necrotic (HR) or mosaic (non-HR).

From 79 TCf18 plants and 108 TCf24 plants, two cuttings per plant were taken for further analysis. After 14 days, one of each pair was inoculated with PVX::*Avr4* and the other with PVX::*Ecp2* (Laugé et al. 1998). Two weeks after inoculation the plants were scored as described above for the PVX::*Avr4* and PVX::*Avr9* tests.

Cuttings of 106 $F_2$Cf18 plants and at least 10 $F_3$Cf18 plants per $F_2$ parent were inoculated with PVX::*Ecp2* and analyzed as described above. If, following infection with PVX::*Ecp2*, a given $F_3$ line contained plants that exhibited mild mosaic symptoms only, we classified the $F_2$ parent as homozygous *cf-ecp2*. If an $F_3$ line contained plants exhibiting necrosis, as well as plants exhibiting only mosaic symptoms, we classified the corresponding $F_2$ parent as heterozygous (*Cf-ECP2*/*cf-ecp2*). If all plants of an $F_3$ line exhibited necrosis we classified the line as homozygous *Cf-ECP2*.

### DNA isolation, CAPS markers and genetic linkage analysis

DNA was isolated from frozen leaf tissue according to a protocol described by Van der Beek et al. (1992). DNA of the parents Moneymaker, Cf18, Cf24 and *L. pimpinellifolium* PI126947 was used to screen for Cleaved Amplified Polymorphic Sequence (CAPS) markers. PCR primers for all CAPS markers on the short arm of chromosome 1 were kindly provided by G. Bonnema (Bonnema et al. 1997, see Table 2), with the exception of the FT33 PCR primers, which were kindly provided by F. Takken (Free University, Amsterdam, The Netherlands). PCR was carried out in a total volume of 50 µl containing 50 ng of DNA, 50 ng of each primer, 100 µM dNTPs, 1× *Taq* buffer (Amersham) and 0.2 U *Taq* polymerase (Amersham). PCR products of the parents were digested with several restriction enzymes (Table 2) to identify which primer pair-restriction enzyme combinations generated CAPS markers. Marker analysis was performed on DNA of pooled leaves from the 106 lines of $F_3$Cf18. Recombination frequencies and LOD values were calculated using the mapping software package JoinMap 2.0 (Stam and Van Ooijen 1996). The segregation of the CAPS marker CT116-*Hha*I was evaluated in the populations TCf18 and TCf24, which were also screened with PVX::*Avr4* and PVX::*Ecp2*.

### Southern analysis

*Bgl*II-digested DNA samples of pooled leaves from 10 plants each of 26 lines of the $F_3$Cf18 population were fractionated on a 0.8% agarose gel and blotted onto a Hybond-N+ membrane. Filters were hybridized with the $^{32}$P-labelled 753-bp *Xho*I-*Sst*I 5′ fragment of the *Cf-9* gene (probe 1: Jones et al. 1994) at 65° C. Blots were washed at 65° C, twice in 2× SSPE, 0.1% SDS, once in 1× SSPE, 0.1% SDS and finally once in 0.2× SSPE, 0.1% SDS.

## Results

### Presence of *Cf-4* and *Cf-9*

Recently, the lines Cf18, Cf20, Cf23, Cf24 and their ancestor *L. pimpinellifolium* PI126947 were shown to harbor *Cf-ECP2* (previously designated lines #1–#4 and ancestor, respectively; Laugé et al. 1998). In an earlier study, Boukema (1980) detected no susceptible recombinants among 100 plants of a testcross population derived from a cross between *L. pimpinellifolium* PI126915 (the original donor of *Cf-9*) and *L. pimpinellifolium* PI126947. This suggested that *L. pimpinellifolium* PI126947 carries either *Cf-9*, or a *Cf* gene which is very closely linked or allelic to *Cf-9*, or both. To test for the presence of *Cf-4* or *Cf-9*, all parents

exhibiting necrosis upon inoculation with PVX::*Ecp2* were inoculated with PVX::*Avr4* and PVX::*Avr9* (results not shown). None of the parents Cf18, Cf20, Cf23 and Cf24, nor their ancestor *L. pimpinellifolium* PI126947, showed necrosis upon inoculation with PVX::*Avr4*. Upon inoculation with PVX::*Avr9*, only *L. pimpinellifolium* PI126947 exhibited necrosis. This indicates the presence of at least two resistance genes, *Cf-9* and *Cf-ECP2*, in this *L. pimpinellifolium* accession, but the breeding lines Cf18, Cf20, Cf23 and Cf24, which are derived from this accession, appear to contain only *Cf-ECP2*.

## Inheritance of *Cf-ECP2*

We hypothesized that *Cf-ECP2*, like all reported *Cf* resistances that show HR, is inherited as a monogenic dominant trait. To test this hypothesis we evaluated a population of 106 $F_2$ plants from a MM × Cf18 cross for the segregation of *Cf-ECP2*. Eighty-one plants showed necrosis and 18 plants mild mosaic symptoms. The remaining seven plants could not be scored unambiguously. This segregation is not significantly different from 3:1 ($P \geq 0.1$) and indicates monogenic dominant inheritance.

To discriminate between homozygous and heterozygous *Cf-ECP2* plants, each $F_2$ plant was selfed and $F_3$ progenies were collected and evaluated for *Cf-ECP2* segregation. The numbers of homozygous *cf-ecp2*, heterozygous, and homozygous *Cf-ECP2* plants were 21, 64 and 21, respectively, corresponding to monogenic inheritance ($P \geq 0.1$). This substantiated the hypothesis that one dominant gene, *Cf-ECP2*, is responsible for resistance to *C. fulvum* conferred by HR-associated recognition of ECP2.

## Linkage tests between *Cf-ECP2* and *Cf-4*

To investigate the possibility that *Cf-ECP2* was linked to the Milky Way cluster we crossed two *Cf-ECP2*-harboring lines (Cf18 and Cf20) to the tomato cultivar MM-Cf4, and the resulting $F_1$ was then crossed to the susceptible cultivar MM. For both populations 24 plants were evaluated for susceptibility or resistance to *C. fulvum* race 0. If *Cf-ECP2* and *Cf-4* are not linked, a 3:1 segregation ratio of resistant to susceptible plants is expected in these populations. In case of linkage, relatively more resistant plants are expected. Three weeks after inoculation with *C. fulvum* race 0, none of the 24 plants from TCf18, and two out of 24 TCf20 plants had sporulating fungi, indicating genetic linkage between *Cf-ECP2* and *Cf-4* ($P \leq 0.005$ and $\leq 0.1$, respectively).

In a second experiment larger testcross populations of TCf18 and TCf24 were inoculated with PVX::*Ecp2* and with PVX::*Avr4* to confirm the presence of the *Cf-ECP2* and *Cf-4* genes, respectively. The plants were divided into four classes: necrotic reaction to both PVX strains, only necrotic to PVX::*Ecp2*, only necrotic to PVX::*Avr4* or necrotic to none of the strains (Table 1). In the case of no linkage the segregation for the four classes should be 1:1:1:1. However, for both the TCf18 and the TCf24 populations segregation deviated significantly from 1:1:1:1 ($P \leq 0.001$), again indicating linkage between *Cf-ECP2* and *Cf-4*. The recombination percentages between these two *Cf* genes in the two populations were 13 and 10, respectively, corresponding to a genetic distance of 11.3 ± 1.8 cM.

## Assignment of *Cf-ECP2* to the short arm of chromosome 1

In order to map *Cf-ECP2* more accurately, we screened the susceptible tomato cultivar Moneymaker and lines harboring *Cf-ECP2* for chromosome 1-specific CAPS markers. With 366 primer-enzyme combinations tested, we detected twelve polymorphisms, representing seven of the twelve loci tested (Table 2). Based on these CAPS markers the size of the introgressed *Cf-ECP2* segment in the Cf18 and Cf24 lines was determined (Fig. 1). The Cf24 line showed the smallest introgression fragment of less than 26 cM, covering only the CT116 and TG184 loci.

For mapping of *Cf-ECP2*, the $F_2$Cf18 population was evaluated for the CAPS markers FT33-*Taq*I, TG236-*Ssp*I, TG184-*Bcl*I and CT116-*Hha*I and for HR-response to PVX::*Ecp2*. Linkage analysis showed that no recombination between *Cf-ECP2* and CT116-*Hha*I could be detected in this population (Fig. 2). To generate an accurate map of the region around *Cf-ECP2*, 74 TCf18 and 102 TCf24 plants were tested for segregation of PVX::*Avr4*, PVX::*Ecp2* and CT116-*Hha*I. Again, CT116-*Hha*I cosegregated completely with *Cf-ECP2* (Fig. 3).

## Southern analysis

As all reported *Cf* genes on the short arm of chromosome 1 are homologous to *Cf-9* (*Hcr9*s), RFLP analysis

**Table 1** Segregation of *Cf-ECP2* and *Cf-4* in two testcross populations using PVX::*Ecp2* and PVX::*Avr4*, respectively

| Population | Neither *Cf* gene[a] | *Cf-ECP2*[b] | *Cf-4*[c] | Both *Cf* genes[d] | Missing[e] | $\chi^{2f}$ |
|---|---|---|---|---|---|---|
| TCf18 | 5 | 36 | 32 | 5 | 1 | 43.5 |
| TCf24 | 7 | 47 | 49 | 4 | 1 | 67.8 |

[a] Number of plants that did not display a necrotic reaction upon inoculation with PVX::*Ecp2* or to PVX::*Avr4*
[b] Number of plants that exhibited necrosis only upon inoculation with PVX::*Ecp2*
[c] Number of plants that exhibited necrosis only upon inoculation with PVX::*Avr4*
[d] Number of plants that exhibited necrotic symptoms upon inoculation with either PVX::*Ecp2* or PVX::*Avr4*
[e] Number of plants that escaped PVX infection
[f] The populations were tested, using a $\chi^2$ test, for 1:1:1:1 segregation, which would indicate that the *Cf-ECP2* and *Cf-4* genes are unlinked. The threshold value ($P \leq 0.001$) was 16.3

**Table 2** Loci, primer sequences, lengths of PCR products and restriction enzymes revealing polymorphisms between *L. esculentum* cv. Moneymaker (MM) and various *Cf-ECP2* lines

| Locus | Primer sequence/reference[a] | Product length (bp) | Enzymes detecting polymorphisms[b] | Total number of enzymes tested |
|---|---|---|---|---|
| CT233 | Bonnema et al. (1997) | 1650 | *Taq*I[3], *Dde*I[3], *Hae*III[3], *Hin*dIII[3] *Nco*I[3] | 43 |
| CT2 | AAGCCTCTAATCAAAAAATGG/ TTCAGTGCAATAATAATGAGGG | 490 | *Hpa*II[1] | 35 |
| TG301 | Bonnema et al. (1997) | 800 | – | 42 |
| CP46 | Bonnema et al. (1997) | 1000 | – | 30 |
| FT33 | AGAAGGATAAAGCTCAACATCGG/ AAGGACATCTGTGGTTCGC | 1100 | *Taq*I[1,2] | 14 |
| TG236 | Bonnema et al. (1997) | 1000 | *Ssp*I[1,2] | 42 |
| CT268 | ATGAAAATGCTCAAATGTTGTTG/ CTTGGATCTTCTGGATTCTACTACC | 300 | – | 4 |
| CT116 | Bonnema et al. (1997) | 1700 | *Hha*I[1,2,3], *Hinf*I[1,2,3] | 25 |
| TG67 | Bonnema et al. (1997) | 1000 | – | 42 |
| TG184 | Bonnema et al. (1997) | 1155 | *Bcl*I[1,2,3] | 18 |
| CT209 | Bonnema et al. (1997) | 600 | – | 26 |
| TG51 | Bonnema et al. (1997) | 1500 | *Hinf*I[1] | 45 |

[a] Forward and reverse primers are separated by a *slash*
[b] The superscripts indicate the source of polymorphism: [1] polymorphism between MM and *L. pimpinellifolium* PI126947; [2] polymorphism between MM and Cf18; and [3] polymorphism between MM and Cf24

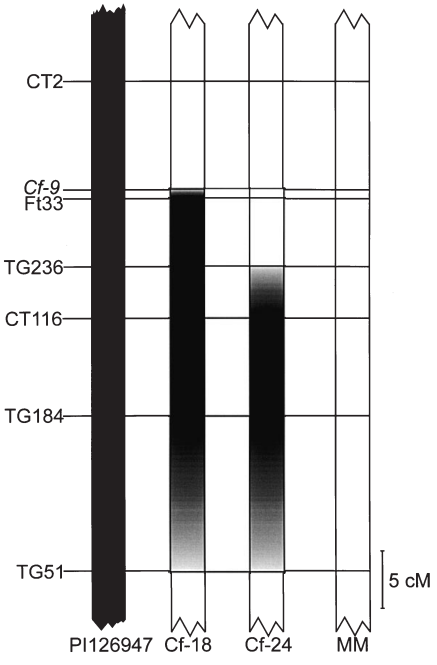


**Fig. 1** Sizes of the introgression fragments in lines harbouring *Cf-ECP2* on the short arm of chromosome 1 in the breeding lines Cf18 and Cf24, based upon the mapping of CAPS between the donor genotype, *L. pimpinellifolium* PI126947 (PI126947) and the susceptible tomato cultivar Moneymaker (MM; see also Table 2). CAPS loci are listed on the left. Distances between loci are estimated based on the present $F_2$ population (FT33, TG236, CT116 and TG184) and other tomato maps (CT2, Tanksley et al. 1992; and TG51, Bonnema et al. 1997). Since *Cf-4* and *Cf-9* are nearly allelic (Thomas et al. 1997), the position of *Cf-9* is the same as the position of *Cf-4* calculated from our experiments (see text). The *open boxes* indicate the Moneymaker (MM) and the *filled boxes* the *L. pimpinellifolium* PI126947 genotype. The positions of recombination events are represented by transitions from black to white or vice versa. The scale (in cM) is indicated by the *vertical bar* on the right

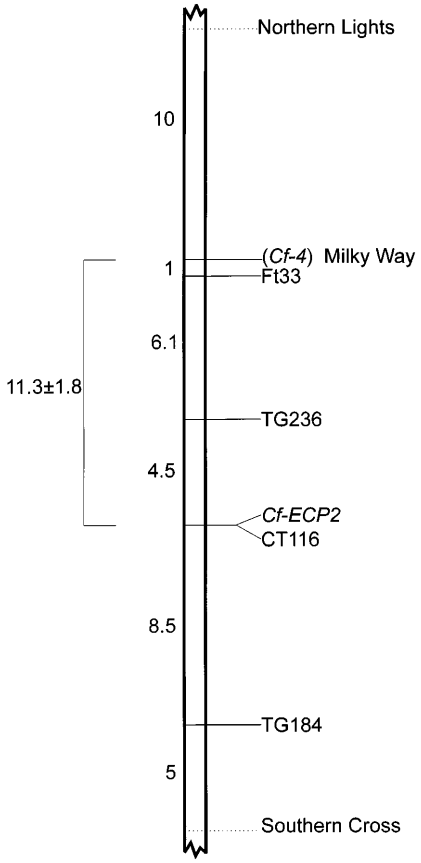


**Fig. 2** Integrated genetic map of the short arm of chromosome 1 around *Cf-ECP2*. Recombination distances between CAPS markers and *Cf-ECP2* are based upon segregation analysis of $F_2$Cf18 and $F_3$Cf18. The position of *Cf-4* relative to *Cf-ECP2* is tentative, and is based upon segregation analysis of 79 TCf18 and 108 TCf24 plants (see text). The map distances (in cM) are indicated on the *left*

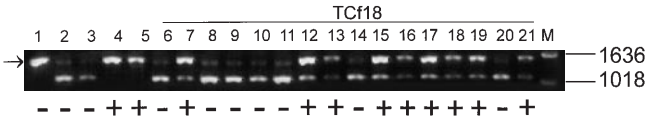


**Fig. 3** PCR products corresponding to the CT116 locus, obtained from 16 members of the TCf18 population and the parental lines, after digestion with *Hha*I and electrophoresis in a 0.8% agarose gel. Lane 1 contains the undigested PCR product of Moneymaker (MM), lanes 2–21 the *HhaI*-digested PCR product of MM (lane 2), MM-Cf4 (lane 3), Cf18 (lane 4), *L. pimpinellifolium* PI126947 (lane 5) and 16 individuals from the TCf18 population (lanes 6–21), respectively. Lane M contains a 1-kb ladder (Life Technologies) as size marker. The *plus* signs indicate necrosis upon inoculation with PVX::*Ecp2*, while a *minus* sign indicates mosaic symptoms upon inoculation with PVX::*Ecp2*. The product of approximately 1550 bp from the Cf18 parent, indicated by the *arrow*, cosegregated with *Cf-ECP2*

with a *Cf-9* probe was performed to detect *Hcr9*s in the lines carrying *Cf-ECP2*: *L. pimpinellifolium* PI126947, Cf18, Cf23 and Cf24 (data not shown). As expected from the results obtained with PVX::*Avr9*, *L. pimpinellifolium* PI126947 showed the 6.7-kb *Bgl*II fragment corresponding to *Cf-9* (Jones et al. 1994). This fragment was not present in Cf18, Cf23 or Cf24. These three breeding lines show one extra *Bgl*II fragment of ≈7.5 kb that was not detected in the near isogenic lines MM, MM-Cf4 and MM-Cf9. In the $F_3$Cf18 population, this *Bgl*II fragment of ≈7.5 kb cosegregated with *Cf-ECP2* (Fig. 4).

## Discussion

Classical breeding for resistance to *C. fulvum* in tomato has resulted in many resistant cultivars, some of which

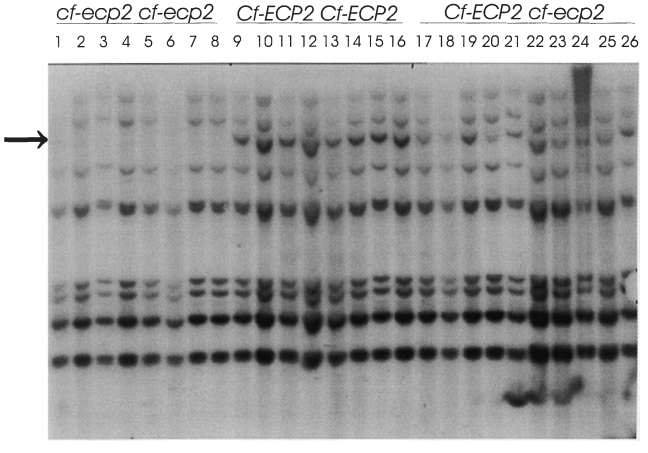


**Fig. 4** Cosegregation of a ≈7.5 kb *Cf-9* hybridizing *Bgl*II fragment with *Cf-ECP2*. Filters were prepared with blotted, *Bgl*II-digested DNA of eight $F_3$Cf18 families originating from homozygous (*cf-ecp2 cf-ecp2*) $F_2$ parents (lanes 1–8), eight $F_3$Cf18 families from homozygous (*Cf-ECP2 Cf-ECP2*) $F_2$ parents (lanes 9–16) and ten $F_3$Cf18 families from heterozygous (*Cf-ECP2 cf-ecp2*) $F_2$ parents (lanes 17–26). The filter was hybridized with the 753-bp *Xho*I-*Sst*I fragment of *Cf-9* (probe 1: Jones et al. 1994). The location of the cosegregating band of ≈7.5 kb is indicated by the *arrow*

contain genes with known specificity while other harbour genes of unknown specificity (Van der Beek et al. 1992). After screening with the fungal protein ECP2, we found that the breeding lines Cf18, Cf20, Cf23 and Cf24, which have been reported to contain different *Cf* genes (Kanwar et al. 1980), all contain one resistance gene that responds to ECP2. Consequently, this gene was designated *Cf-ECP2* (Laugé et al. 1998). Although they apparently contain the same resistance gene, these *Cf* lines have been assigned different names – Cf18, Cf20, Cf23 and Cf24 (Kanwar et al. 1980) – based on phenotypic differences in the resistance reaction to *C. fulvum*. Similarly, Gerlagh et al. (1989) showed that *Cf-4* and *Cf-8* are indistinguishable with respect to their interaction with *C. fulvum* races. These results indicate that phenotypic differences in the resistance reaction are not sufficient to justify the designation of a new *Cf* gene. For the correct designation of a new *Cf* gene, it is essential either to find races of *C. fulvum* that differentiate between this new *Cf* gene and all previously described *Cf* genes or to characterize it by its specific interaction with a new avirulence gene like *Ecp2*. Based on these results and considerations we propose not to use the designations Cf18, Cf20, Cf23 and Cf24 anymore, but to replace them all by the term *Cf-ECP2*.

*Cf-ECP2* might confer longer lasting resistance than other known *Cf* genes that have been used in breeding programs and have rapidly lost their effectiveness, as the *Cf-ECP2* gene product recognizes the fungal protein ECP2, which is essential for pathogenesis (Laugé et al. 1998).

Using PVX::*Avr9*, it was shown that the original donor of *Cf-ECP2*, *L. pimpinellifolium* PI126947, harbors not only *Cf-ECP2*, but also a gene for the recognition of AVR9. Jones et al. (1994) have isolated *Cf-9*, which originates from another *L. pimpinellifolium* accession, PI126915 (Tigchelaar 1984; Balint-Kurti et al. 1994). Since Boukema (1980) found no susceptible recombinants among the offspring of a testcross between the *L. pimpinellifolium* accessions PI126915 and PI126947, their resistance genes must be closely linked, allelic or identical. PI126915 only harbours one dominant *Cf* gene (Boukema 1980), which is *Cf-9* (Tigchelaar 1984). Since *Cf-4* and *Cf-9* are nearly allelic (Thomas et al. 1997), and the distance between *Cf-ECP2* and *Cf-4* is 11.3 ± 1.8 cM, *Cf-ECP2* is not the resistance gene which is closely linked or allelic to the *Cf-9* gene from PI126915. Therefore, the second resistance gene present in PI126947, which recognizes AVR9, is closely linked or allelic to the *Cf-9* locus of PI126915. The fact that both lines are *L. pimpinellifolium* accessions suggests that these *Cf-9* genes are either identical or originate from a common ancestor. If they are derived from a common ancestor, it will be interesting to isolate and sequence the *Cf-9* gene from PI126947 to see whether mutations are allowed without loss of AVR9 recognition.

The previously characterized *Cf* genes which are located on the short arm of chromosome 1, *Cf-4* and *Cf-9*, are present in the Milky Way cluster (Parniske et al.

1998). As concluded from the testcross analysis, *Cf-ECP2* is linked to *Cf-4* at a distance of 11.3 ± 1.8 cM. This indicates that *Cf-ECP2* is not part of the Milky Way cluster. Subsequently, *Cf-ECP2* was mapped accurately by using CAPS markers that discriminate between *L. pimpinellifolium* PI126947 and Moneymaker (Table 2). By using these CAPS markers and the PVX::*Avr9* test, we determined the length of the introgression fragments in the breeding lines Cf18 and Cf24, which are derived from *L. pimpinellifolium* PI126947. Cf24 has the smallest introgressed region and, like Cf18, does not contain the Milky Way cluster of the *L. pimpinellifolium* donor. The size of the introgression fragments is less than 33 cM in Cf18 and less than 26 cM in Cf24, which suggests a limited linkage drag (Young and Tanksley 1989). From the length of the smallest introgression fragment, which is present in Cf24, it can be concluded that *Cf-ECP2* is located between TG236 and TG51. Indeed, *Cf-ECP2* was mapped at the locus CT116, which lies between TG236 and TG184 (Fig. 2). In most populations studied, these markers reside in a cluster, referred to as "the TG236 cluster" (Tanksley et al. 1992; Balint-Kurti et al. 1994). However, by using an $F_2$ population derived from an interspecific cross between *L. esculentum* and *L. peruvianum*, Bonnema et al. (1997) showed that the markers present in the TG236 cluster span a region of approximately 15 cM, with a distance of 12.6 cM between TG236 and TG184. In the present $F_2$ population we found a similar distance of 13.7 cM between TG236 and TG184. *Cf-ECP2* cosegregated completely with CT116 in all populations tested ($n = 282$). Parniske et al. (1998) showed the presence of at least one *Hcr9* cluster proximal to the TG236 cluster, designated Southern Cross. However, no *Hcr9* clusters cosegregating with the TG236 cluster (containing CT116) were reported. Consequently, *Cf-ECP2* is located at an as yet unidentified *Cf* locus and is the first functional *Cf* gene located on the short arm of chromosome 1 that is not part of the Milky Way cluster. Moreover, Southern analysis showed that a *Cf-9* hybridizing band of ≈7.5 kb cosegregates with *Cf-ECP2*. Therefore, it is likely that *Cf-ECP2* is homologous to *Cf-9* (*Hcr9*), like all other *Cf* genes on the short arm of chromosome 1 reported so far. We propose to designate this *Hcr9* locus Orion.

Although no other *Hcr9*s have been observed to cosegregate with *Cf-ECP2*, it cannot be excluded that other *Hcr9*s might be present at Orion, but escaped detection because they are not polymorphic with respect to *Hcr9*s from other clusters in the *Bgl*II digest. Similarly, the ≈7.5-kb *Bgl*II fragment does not necessarily correspond to *Cf-ECP2*.

Parniske et al. (1998) suggested that new *Cf* clusters might arise by chromosomal duplication or retroposition events. The finding of *Cf-ECP2* at a position where no *Cf* homologues have been reported before might give us new insights into the molecular processes that lead to the development of new resistance specificities. If the wild ancestor of *Cf-ECP2*, *L. pimpinellifolium* PI126947, also harbors the *Cf-9* gene of PI126915, the isolation and the genetic organization of *Cf-ECP2* will reveal how these *Cf* genes and *Cf* clusters have been evolving. Bonnema et al. (1997) showed in an $F_2$ population derived from a cross between *L. esculentum* and *L. peruvianum* LA2157 that in the region of the CT116 locus 1 cM corresponds to a physical distance of maximally 52 kb. Since genetic distances are similar in the $F_2$ population that we have used, and *Cf-ECP2* is likely to be a *Hcr9*, the mapping of *Cf-ECP2* near CT116 will allow us to exploit the homology to *Cf-9* as well as this close linkage to CT116 to isolate this resistance gene.

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## References

Bailey DL, Kerr EA (1964) *Cladosporium fulvum* race 10 and resistance to it in tomato. Can J Bot 42:1555–1558

Balint-Kurti PJ, Dixon MS, Jones DA, Norcott KA, Jones JDG (1994) RFLP linkage analysis of the *Cf-4* and *Cf-9* genes for resistance to *Cladosporium fulvum* in tomato. Theor Appl Genet 88:691–700

Bonnema G, Schipper D, Van Heusden S, Zabel P, Lindhout P (1997) Tomato chromosome 1: high-resolution genetic and physical mapping of the short arm of an interspecific *Lycopersicon esculentum* × *L. peruvianum* cross. Mol Gen Genet 253:455–462

Boukema IW (1980) Research on allelism of genes for resistance to *Cladosporium fulvum* Cke. (*Fulvia fulva*) in tomato. Meeting on *Cladosporium fulvum* Cke in Tomato, 26–27 February, 1980

Dickinson MJ, Jones DA, Jones JDG (1993) Close linkage between the *Cf-2*/*Cf-5* and *Mi* resistance loci in tomato. Mol Plant-Microbe Interact 6:341–347

Flor HH (1942) Inheritance of pathogenicity in *Melampsora lini*. Phytopathol 32:653–669

Gerlagh M, Lindhout WH, Vos I (1989) Allelic test proves genes $Cf_4$ and $Cf_8$ for resistance to *Cladosporium fulvum* (*Fulvia fulva*) on tomato to be undistinguishable. Neth J Plant Pathol 95:357–359

Hammond-Kosack KE, Staskawicz BJ, Jones JDG, Baulcombe DC (1995) Functional expression of a fungal avirulence gene from a modified potato virus X genome. Mol Plant-Microbe Interact 8:181–185

Jones DA, Thomas CM, Hammond-Kosack KE, Balint-Kurti PJ, Jones JDG (1994) Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging. Science 266:789–793

Joosten MHAJ, Cozijnsen TJ, De Wit PJGM (1994) Host resistance to a fungal tomato pathogen lost by a single base-pair change in an avirulence gene. Nature 367:384–386

Joosten MHAJ, Vogelensang R, Cozijnsen TJ, Verberne MC, De Wit PJGM (1997) The biotrophic fungus *Cladosporium fulvum* circumvents *Cf-4*-mediated resistance by producing unstable AVR4 elicitors. Plant Cell 9:367–379

Kanwar JS, Kerr EA, Harney PM (1980) Linkage of the *Cf-12* to *Cf24* genes for resistance to tomato leaf mold *Cladosporium fulvum* Cke. Rep Tomato Genet Coop 30:22–23

Laterrot H (1986) Race 2.5.9, a new race of *Cladosporium fulvum* (*Fulvia fulva*) and sources of resistance in tomato. Neth J Plant Path 92:305–307

Laterrot H, Gerlagh M, Ester A, Stamova L (1985) Race 2.5, a new race of *Cladosporium fulvum* (*Fulvia fulva*) on tomato. Neth J Plant Pathol 91:45–47

Laugé R, Joosten MHAJ, Van den Ackerveken GFJM, Van den Broek HWJ, De Wit PJGM (1997) The *in planta*-produced extracellular proteins ECP1 and ECP2 of *Cladosporium fulvum* are virulence factors. Mol Plant-Microbe Interact 10:725–734

Laugé R, Joosten MHAJ, Haanstra JPW, Goodwin PH, Lindhout P, De Wit PJGM (1998) Successful search for a resistance gene in tomato targeted against a virulence factor of a fungal pathogen. Proc Natl Acad Sci USA 95:9014–9018

Lindhout P, Korta W, Cislik M, Vos I, Gerlagh T (1989) Further identification of races of *Cladosporium fulvum* (*Fulvia fulva*) on tomato originating from the Netherlands, France and Poland. Neth J Plant Pathol 95:143–148

Parniske M, Wulff BBH, Bonnema G, Thomas CM, Jones DA, Jones JDG (1998) Homologues of the *Cf-9* disease resistance gene (*Hcr9*s) are present at multiple loci on the short arm of tomato chromosome 1. Mol Plant-Microbe Interact 12:93–102

Stam P, Van Ooijen JW (1996) JoinMap version 2.0: software for the calculation of genetic linkage maps. CPRO-DLO, Wageningen

Stevens MA, Rick CM (1988) Genetics and breeding. In: Atherton JG, Rudich J (eds) The tomato crop. Chapman and Hall, London, pp 35–109

Takken FLW, Schipper D, Nijkamp HJJ, Hille J (1998) Identification and *Ds*-tagged isolation of a new gene at the *Cf-4* locus of tomato involved in disease resistance to *Cladosporium fulvum* race 5. Plant J 14:401–411

Tanksley SD, et al (1992) High density molecular linkage maps of the tomato and potato genomes. Genetics 132:1141–1160

Thomas CM, Jones DA, Parniske M, Harrison K, Balint-Kurti PJ, Hatzixanthis K, Jones JDG (1997) Characterization of the tomato *Cf-4* gene for resistance to *Cladosporium fulvum* identifies sequences that determine recognitional specificity in *Cf-4* and *Cf-9*. Plant Cell 9:2209–2224

Tigchelaar EC (1984) Collections of isogenic tomato stocks. Rep Tomato Genet Coop 34:55–57

Van den Ackerveken GFJM, De Wit PJGM (1994) The *Cladosporium*-tomato interaction: a model system for fungus-plant specificity. In: Kohmoto K, Singh RP, Singh US, Ziegler R (eds) Pathogenesis and host specificity in plant diseases. Pergamon Press, Oxford, pp 145–160

Van den Ackerveken GFJM, Van Kan JAL, De Wit PJGM (1992) Molecular analysis of the avirulence gene *avr9* of *Cladosporium fulvum* fully supports the gene-for-gene hypothesis. Plant J 2:359–366

Van den Ackerveken GFJM, Van Kan JAL, Joosten MHAJ, Muisers JM, Verbakel HM, De Wit PJGM (1993) Characterization of two putative pathogenicity genes of the fungal tomato pathogen *Cladosporium fulvum*. Mol Plant-Microbe Interact 6:210–215

Van der Beek JG, Verkerk R, Zabel P, Lindhout P (1992) Mapping strategy for resistance genes in tomato based on RFLPs between cultivars: *Cf9* (resistance to *Cladosporium fulvum*) on chromosome 1. Theor Appl Genet 84:106–112

Van Kan JAL, van den Ackerveken GFJM, de Wit PJGM (1991) Cloning and characterization of cDNA of avirulence gene *Avr9* of the fungal pathogen *Cladosporium fulvum*, causal agent of tomato leaf mold. Mol Plant-Microbe Interact 4:52–59

Young ND, Tanksley SD (1989) RFLP analysis of the size of chromosomal segments retained around the *Tm-2* locus of tomato during backcross breeding. Theor Appl Genet 77:353–359