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ORIGINAL PAPER

The *Solanum pimpinellifolium Cf-ECP1* and *Cf-ECP4* genes for resistance to *Cladosporium fulvum* are located at the *Milky Way* locus on the short arm of chromosome 1

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Abstract The interaction between tomato and the leaf mould pathogen *Cladosporium fulvum* is an excellent model to study gene-for-gene interactions and plant disease resistance gene evolution. Most *Cf* genes were introgressed into cultivated tomato (*Solanum lycopersicum*) from wild relatives such as *S. pimpinellifolium* and novel *Cf-ECP* genes were recently identified in this species. Our objective is to isolate *Cf-ECP1, Cf-ECP2, Cf-ECP4* and *Cf-ECP5* to increase our understanding of *Cf* gene evolution, and the molecular basis for recognition specificity in Cf proteins. The map locations of *Cf-ECP2* and *Cf-ECP5* have been reported previously and we report here that *Cf-ECP1* and *Cf-ECP4* map to a different locus on the short arm of chromosome 1. The analysis of selected recombinants and allelism tests showed both genes are located at *Milky Way* together with *Cf-9* and *Cf-4*. Our results emphasise the importance of this locus in generating novel *Cf* genes for resistance to *C. fulvum*. Candidate genes for *Cf-ECP1* and *Cf-ECP4* were also identified by DNA gel blot analysis of bulked segregant pools. In addition, we generated functional cassettes for expression of the *C. fulvum* ECP1, ECP2, ECP4 and ECP5 proteins using recombinant *Potato Virus X*, and three *ECP*s were also expressed in stable transformed plants. Using marker-assisted selection we have also identified recombinants containing *Cf-ECP1*, *Cf-ECP2*, *Cf-ECP4* or *Cf-ECP5 in cis* with a linked T-DNA carrying the non-autonomous *Zea mays* transposon *Dissociation*. Using these resources it should now be possible to isolate all four *Cf-ECP*s using transposon tagging, or a candidate gene strategy.

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Introduction

The interaction between cultivated tomato (*Solanum lycopersicum*) and the leaf mould pathogen *Cladosporium fulvum* is an excellent model to study gene-for-gene interactions and plant disease resistance gene evolution (reviewed in Rivas and Thomas 2005). Resistance genes like *Cf-9* and *Cf-4* are members of a complex genetic locus on the short arm of chromosome 1 that were originally identified in the wild tomato species *S. pimpinellifolium* and *S. hirsutum*, and bred into cultivated tomato (Stevens and Rick 1988; Rivas and Thomas 2005). All *Cf* genes characterized to date encode type I trans-membrane extracellular leucine-rich repeat glycoproteins, which lack an obvious cytoplasmic signalling domain (Rivas and Thomas 2005). Cf proteins activate a hypersensitive response (HR) upon recognition of genetically defined *C. fulvum* avirulence (Avr) proteins, but the molecular mechanism of Avr protein perception, and activation of the HR, have yet to be determined (Rivas and Thomas 2005).

Several *Cf* genes from the *Milky Way* (*MW*) locus on the short arm of tomato chromosome 1, including *Cf-4*, *Hcr9-4E*,

Cf-9 and *Hcr9-9B*, have been cloned and extensively characterised (Jones et al. 1994; Thomas et al. 1997; Takken et al. 1999; Panter et al. 2002) and these studies form the basis for our current understanding of *Cf* gene evolution. The *MW* locus is composed of variable numbers of tandemly duplicated *h*omologues of *Cladosporium r*esistance gene *Cf-9* (*Hcr9*s) and one mechanism that generates *Cf* gene variation is sequence exchange between *Hcr9* paralogues (Parniske et al. 1997; Kruijt et al. 2004). The isolation and characterisation of additional *Cf* genes at *MW* would significantly increase our understanding of the organisation and evolution of *Cf* genes at *MW*, together with the molecular basis for Cf protein recognition specificity.

*C. fulvum e*xtracellular *p*roteins (ECPs) are secreted into the leaf apoplast during infection and they share several features with *C. fulvum* Avr proteins, including their low molecular weight. The mature processed forms of ECPs contain an even number of cysteine residues that appear to function in disulphide bridge formation (Van Den Ackerveken et al. 1993; Lauge et al. 2000) but some of these are dispensible, at least in terms of their necrosis-inducing activity (Luderer et al. 2002). Functional analysis of a limited number of *S. pimpinellifolium* accessions was sufficient to identify five novel *Cf* genes (*Cf-ECP1*, *Cf-ECP2*, *Cf-ECP3*, *Cf-ECP4* and *Cf-ECP5*) that trigger an HR in response to the *C. fulvum* ECP1, ECP2, ECP3, ECP4 and ECP5 proteins (Lauge et al. 1998, 2000). Genetic mapping showed *Cf-ECP2* and *Cf-ECP3* defined a new complex locus for *C. fulvum* resistance at *Orion* (*OR*) on the short arm of chromosome 1 (Haanstra et al. 1999; Yuan et al. 2002) and the mapping of *Cf-ECP5* also defined a new locus, located 3 cM proximal to *MW*, which was designated *Aurora* (*AU*; Haanstra et al. 2000). Solanum germplasm is therefore an important source of novel *Cf* genes (Rivas and Thomas 2005).

Mapping of the *S. pimpinellifolium Cf-ECP1* and *Cf-ECP4* genes has not been reported previously and we show here, by molecular analysis of selected recombinants and allelism tests, that both genes are located at *MW* along with *Cf-9* and *Cf-4*. We used DNA gel blots to identify candidate *Hcr9*s for *Cf-ECP1* and *Cf-ECP4*. As a preliminary step towards gene isolation by transposon tagging we generated lines containing *Cf-ECP1*, *Cf-ECP2*, *Cf-ECP4* or *Cf-ECP5* in *cis* with a linked T-DNA carrying the non-autonomous *Zea mays* transposon *Dissociation* (*Ds*) (Rommens et al. 1992). We also constructed cassettes for the *in planta* expression of ECP1, ECP2, ECP4 and ECP5 proteins using Agrobacterium-mediated delivery of recombinant *Potato Virus X* (PVX), or stable transformed plants. Together, these resources will facilitate the isolation and characterisation of all four *Cf-ECP* genes using a candidate gene approach, or transposon tagging with *Ds*.

Materials and methods

Tomato stocks

Cf-ECP stocks were obtained from Dr. MHAJ Joosten and Dr. P Lindhout at the University of Wageningen, The Netherlands; *S. pimpinellifolium* containing *Cf-ECP1* (LA1547) and *Cf-ECP4* (LA1683); *S. lycopersicum* containing the introgressed *S. pimpinellifolium* genes *Cf-ECP2* (Ontario 7518) and *Cf-ECP5* (G1.1161) as reported by Haanstra et al. (1999, 2000). Other *S. lycopersicum* lines were supplied by Matthew Smoker at The Sainsbury Laboratory, UK; *S. lycopersicum* var. Moneymaker (Cf0), line FT33 (Rommens et al. 1992), and line M18, which contains a *Ds*-tagged allele of *Cf-9* (*Cf-9*:*Ds*^{M18}; Jones et al. 1994).

ECP1, ECP2, ECP4 and ECP5 expression constructs

Oligonucleotides were obtained from MWG BIOTECH and are available on request. Sequences encoding *C. fulvum* ECP1, ECP2, ECP4 and ECP5 were constructed as described previously (Thomas et al. 1997). Each gene was amplified in two parts from *C. fulvum* race five genomic DNA. Complete *ECP* coding sequences were reconstituted using overlap PCR. Oligonucleotides to the 3′ end of each reading frame included a *Bam*HI sequence for cloning into vector pSPNae (Thomas et al. 1997). All four sequences were ligated into *Nae*I/*Bam*HI digested pSPNae to generate in-frame fusions of each *ECP* with sequences encoding the *Nicotiana tabacum* PR1A signal peptide.

PR1A:ECP fusions were cloned as *Cla*I/*Bam*HI fragments into pSLJ4K1 (Thomas et al. 1997) fusing the *PR1A:ECP* sequences to the cauliflower mosaic virus 35S promoter, and the *Agrobacterium tumefaciens* nopaline synthase (*nos*) transcription terminator. *PR1A:ECP* sequences were excised as *Eco*RI/*Hin*dIII fragments and cloned into pSLJ7291 (Jones et al. 1992) for transformation of *S. lycopersicum*. *PR1A:ECP* sequences were also cloned as *Cla*I/*Sa*lI fragments into pGR107, which contains a cDNA copy of PVX. Recombinant ECP clones were transferred into *A. tumefaciens* GV3101 by electroporation.

DNA sequence analysis

Sequences of *PR1A:ECP* clones were confirmed using an ABI BigDye sequencing kit (Applied Biosystems). Reaction products were electrophoresed on an Applied Biosystems 377 automated sequencer and sequences were assembled using UNIX-based versions of the Staden Program Package.

Generation of tomato transformants

Binary vector plasmids were mobilized from *Escherichia coli* DH5α into *A. tumefaciens* LBA4404 using tri-parental mating, as described by Jones et al. (1992). The transformation of tomato cotyledons (*S. lycopersicum* var. Moneymaker), and plant regeneration procedures were performed essentially as described by Dixon et al. (1996).

Delivery of recombinant PVX using Agrobacterium infiltration

A. tumefaciens GV3101 clones containing *PR1A:ECP*s were grown to saturation at 28°C in liquid culture containing 50 μg/ml kanamycin. Bacteria were pelleted at 5,000*g* for 10 min at 15°C and resuspended in two times the original volume of a solution containing 1× Murashige and Skoog salts (Duchefa), 2% w/v sucrose and 150 μM acetosyringone. Bacteria were incubated at room temperature for 2–3 h and then infiltrated into tomato cotyledons.

Construction of Cf-ECP1 and Cf-ECP4 bulked segregant pools

Bulked segregant pools from Cf-ECP1 × Cf0 ($F_2$1E) and Cf-ECP4 × Cf0 F_2 populations ($F_2$4E) were constructed from c.80 F_2 seedlings. A single cotyledon was harvested from all F_2s in each population to constitute the *Cf-ECP*/+ bulk. Each F_2 population was then infected with PVX:*ECP1* or PVX:*ECP4* and scored for systemic necrosis at 14 days post-infection. Plant material from wildtype seedlings in each population was pooled and used to prepare DNA for the +/+ bulk.

Nucleic acid preparations

DNA was prepared from Cf-ECP lines and for bulked segregant analysis as follows. Cotyledons or young leaves (2.5 g) were ground in liquid N_2 and transferred to a tube containing 5 ml phenol–chloroform–isoamyl alcohol (25:24:1) and 7.5 ml of NTES buffer (100 mM NaCl, 10 mM Tris–HCl, pH 7.5, 1 mM EDTA and 1% w/v SDS). Samples were centrifuged at 8,000 rpm in a Sorvall SS34 rotor at 4°C for 10 min. The supernatant was adjusted to 150 mM NaAc pH 6 and nucleic acids were precipitated by adding an equal volume of isopropanol. Samples were centrifuged at 8,000 rpm in an SS34 rotor for 15 min at 4°C. Nucleic acids were resuspended in sterile water and LiCl was added to the supernatant to 2 M, incubated on ice for 1 h and centrifuged at 8,000 rpm in an SS34 rotor for 15 min at 4°C. DNA was ethanol precipitated from the supernatant.

DNA gel blot analysis

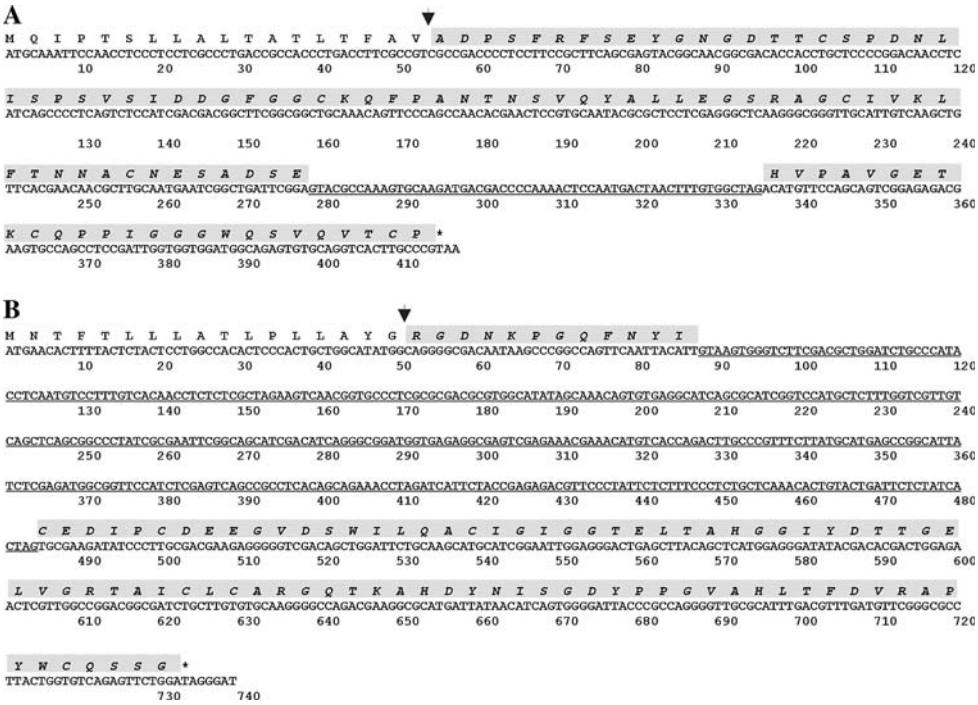
Two to 5 μg of tomato DNA was digested at 37°C for 16 h, extracted with phenol–chloroform and ethanol precipitated. DNA was electrophoresed for 20 h at 2.5 v/cm in 0.8% w/v agarose gels in a vertical gel apparatus using 40 mM Tris–acetate, pH 7.9, 1 mM EDTA as running buffer. Nucleic acids were transferred to Hybond-N membrane (Amersham) and cross-linked by irradiation with UV light. A 1.5 kbp probe was PCR amplified from a *Cf-9* cDNA clone (Jones et al. 1994) using primers Cf95′ (AAAGCAAACA TTTCTTGATTTCTT) and Cf9F2 (GCTTGGAATATGA CCTTCAAATCTG). Filters were hybridised with ^{32}P-labelled probes at 65°C for 16–20 h, and washed four times for 15 min in 2 × SSC (1× SSC is 0.15M sodium chloride, 0.015 M sodium citrate) and 1% w/v SDS at 65°C, and for 30 min in 0.2× SSC and 0.1% w/v SDS at 65°C.

Results

Construction of ECP expression cassettes and functional analysis using recombinant PVX

Genomic or cDNA clones encoding *C. fulvum* ECP1, ECP2, ECP4 and ECP5 were isolated previously based upon their partial amino acid sequences (Van Den Ackerveken et al. 1993; Lauge et al. 2000). Genomic sequences of *ECP4* and *ECP5* have not been reported and these were amplified from *C. fulvum* race 5 DNA using primers to the reported cDNA sequences (Lauge et al. 2000). Like *ECP2*, the coding regions of *ECP4* and *ECP5* are interrupted by a single intron (Fig. 1). *ECP* cassettes encoding the mature secreted forms of each ECP were generated by overlap PCR and fused in-frame to sequences encoding the *N. tabacum* PR1A signal peptide, which can target proteins to the leaf apoplast *in planta*. The four *PR1A:ECP* cassettes were also cloned into a binary vector containing a cDNA copy of PVX for Agrobacterium-mediated delivery of *C. fulvum* Avr and ECP proteins (Lauge et al. 2000; Luderer et al. 2002).

To determine if *PR1A:ECP* sequences can induce a *Cf*-dependent HR suspensions of *A. tumefaciens* were infiltrated into a range of *Cf-ECP* genotypes (Fig. 2). In most cases recombinant PVX only resulted in symptoms characteristic of wildtype PVX infection (Fig. 2a). However, each *PVX:ECP* induced necrotic lesions in systemically infected leaves of seedlings expressing its cognate *Cf-ECP* (Fig. 2). Leaf epinasty was followed by development of necrotic lesions that spread systemically, resulting in seedling death. These features are similar to other reports of PVX-mediated expression of *C. fulvum* ECP and AVR proteins in tomato (Hammond-Kosack et al. 1995; Thomas et al. 1997; Lauge



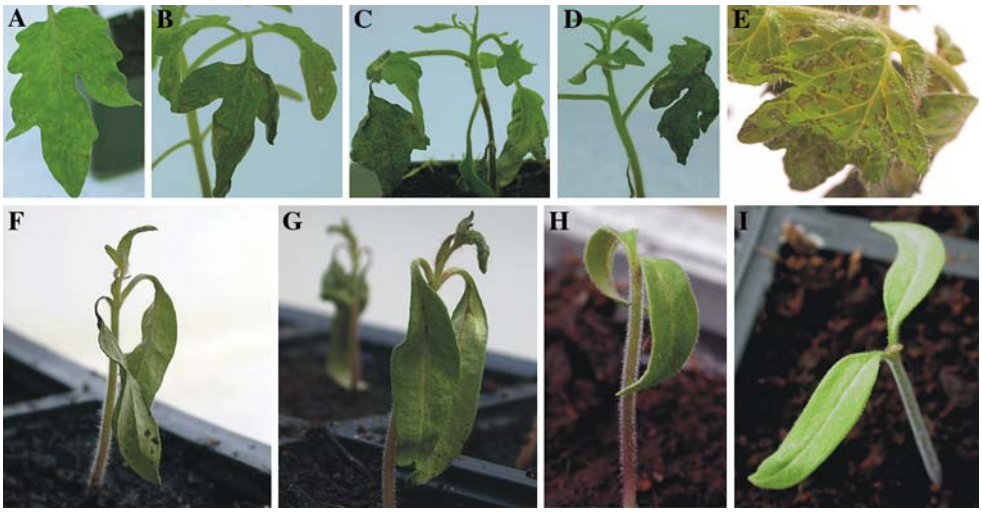


Fig. 1 Genomic sequences of *C. fulvum ECP4* and *ECP5*. The coding regions of *ECP4* (**a**) and *ECP5* (**b**) were amplified using primers to the 5′ and 3′ ends of their corresponding cDNAs. Intron sequences are shown underlined and each exhibits the characteristic 5′ donor and 3′ acceptor sequences of eukaryotic introns. The amino acid sequence of each protein is shown above the nucleotide sequence. The predicted proteolytic cleavage sites of each signal peptide sequence (Lauge et al. 2000) is indicated with an *arrowhead*, and the mature secreted protein is shown *italicised* and highlighted in *grey*

Fig. 2 PVX-mediated delivery of *PR1A:ECP* sequences and stable expression in *S. lycopersicum* induces a HR on tomato genotypes expressing the cognate *Cf-ECP* gene. **a** Symptoms of wild type PVX infection (vein clearing and chlorosis) on leaves lacking *Cf-ECP* genes. **b** Leaf epinasty and necrotic lesions on leaves of Cf-ECP1 plants (LA1547) induced by PVX:*ECP1*. **c** Necrotic lesions on leaves of Cf-ECP2 plants (Ontario 7518) induced by PVX:*ECP2*. **d** PVX:*ECP4*-induced leaf epinasty and necrosis in Cf-ECP4 plants (LA1683) **e** Developing necrotic lesions on Cf-ECP5 plants (G1.1161) induced by PVX:*ECP5*. **f** Seedling lethality in progeny from a cross between an ECP2-expressing line (UEA721) and *Cf-ECP2* at 18 dpg. **g** Lethality in progeny from a cross between an ECP4-exprssing line (UEA 678) and *Cf-ECP4* at 18 dpg. **h** Progeny expressing *ECP5* and *Cf-ECP5* show wilting of the cotyledons and lack leaf primordia compared to wildtype segregants (**i**)

et al. 2000; Luderer et al. 2002) and demonstrates all four *PVX:ECP* sequences are functional *in planta*.

Analysis of PR1A:ECP expression in transformed plants

Stable expression of *C. fulvum Avr* genes in tomato can be achieved in genotypes lacking the cognate *Cf* gene (Hammond-Kosack et al. 1994; Thomas et al. 1997). The F_1 progeny from crosses between *Avr*-expressing plants and lines containing the corresponding *Cf* gene exhibit seedling lethality due to activation of a systemic HR at about 12 days post-germination (Hammond-Kosack et al. 1994; Thomas et al. 1997).

We generated ten primary transformants for each *PR1A:ECP* construct in the *S. lycopersicum* cultivar ‘‘Moneymaker’’ (Cf0). Approximately 25% of transformants

were sterile polyploids and c. 30% lacked the *ECP* transgene (results not shown). The remaining transformants were used as female parents in test crosses to Cf-ECP lines to identify transformants expressing functional ECPs (Table 1). Genetic analysis of self progeny (Table 1) from each transformant enabled us to identify active lines containing single copy T-DNAs for *ECP2* (UEA721), *ECP4* (UEA678 and UEA682), and *ECP5* (UEA726, UEA729 and UEA730). No transformants expressing *ECP1* were obtained in this study.

Development of the seedling lethal phenotype induced by *ECP2* and *ECP4* was similar to that reported for the *C. fulvum Avr9* and *Avr4* genes in Cf-9 and Cf-4 plants (Hammond-Kosack et al. 1994; Thomas et al. 1997). Wilting of the cotyledons was observed at 14 days post-germination (dpg) followed by necrosis in the cotyledons and true leaves at 18 dpg (Fig. 2f, g). The seedling lethal phenotype induced in all three *ECP5*-expressing plants was distinct from *ECP2* and *ECP4* (Fig. 2h). In a segregating population seedlings expressing *Cf-ECP5* and *ECP5* were stunted at 14 dpg compared to wildtype segregants and showed wilting of the cotyledons. The seedlings failed to produce true leaves (Fig. 2h) and seedling death was observed at approximately 21 dpg (not shown).

The Cf-ECP1 and Cf-ECP4 genes are located on the short arm of chromosome 1

Our preliminary analysis showed *Cf-ECP1* and *Cf-ECP4* are located on chromosome 1 (results not shown). To map the genes more accurately we prepared bulked segregant pools from Cf-ECP1 × Cf0 and Cf-ECP4 × Cf0 F_2 populations ($F_2$1E and $F_2$4E) for marker analysis. We used cleaved amplified polymorphic sequence (CAPS) markers located at or close to *MW* (CT116, TG236, LoxR and CP46), which have been characterised previously (Balint-Kurti et al. 1994; Haanstra et al. 1999; Wulff et al. 2004).

LoxR is derived from sequences at the 3′ end of the lipoxygenase *LoxR* gene at the distal end of *MW* (Wulff et al. 2004) and the Cf-ECP1 allele ($LoxR^{Cf\text{-}ECP1}$) lacks a site for *Hin*cII (Fig. 3a). CAPS analysis of *Cf-ECP1*/+ and +/+ bulks showed $LoxR^{Cf\text{-}ECP1}$ cosegregates with *Cf-ECP1* (Fig. 3a). The *S. lycopersicum* allele of TG236 ($TG236^{lyc}$), which is located proximal to *MW* (Balint-Kurti et al. 1994; Haanstra et al. 1999, 2000) contains a site for *Ssp*I, which is absent from the Cf-ECP4 allele ($TG236^{Cf\text{-}ECP4}$; see Fig. 3b). CAPS analysis of *Cf-ECP4*/+ and +/+ bulks showed $TG236^{Cf\text{-}ECP4}$ cosegregates with *Cf-ECP4*. These data show *Cf-ECP1* and *Cf-ECP4* are both located on the short arm of chromosome 1.

Table 1 Genetic analysis of ECP transgenics

Stock	Transgene	Kan^R; Kan^S [a]	Genotype of TC parent	Ratio of necrotic: wildtype TC progeny
UEA721	ECP2	11:5 (1)	*Cf-ECP2*	16:13
UEA678	ECP4	20:5 (1)	*Cf-ECP4*	15:11
UEA682	ECP4	14:4 (1)	*Cf-ECP4*	11:14
UEA726	ECP5	38:11 (1)	*Cf-ECP5*	10:14
UEA729	ECP5	16:5 (1)	*Cf-ECP5*	15:12
UEA730	ECP5	14:3 (1)	*Cf-ECP5*	9:11

[a] The ratio of kanamycin resistant (Kan^R) to susceptible (Kan^S) progeny is shown and the number of predicted T-DNA loci based on a χ^2 goodness-of-fit test to a 3:1 ratio is shown in parentheses

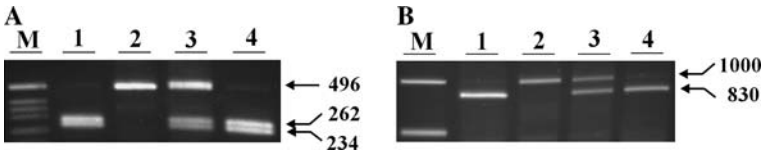


Fig. 3 CAPS analysis of $F_2$1E and $F_2$4E bulked segregant pools. **a** PCR amplification with LoxR-F1 and LoxR-R1 primers generates a 496 bp product from Cf0 and Cf-ECP1. Only the Cf0 allele (*lane 1*) contains a target for *Hin*cII, which generates cleavage products of 262 and 234 bp. The *Hin*cII site is absent from the Cf-ECP1 allele (*lane 2*). The Cf-ECP1/+ bulk (*lane 3*) contains both alleles but the Cf-ECP1 allele is absent from the +/+ bulk (*lane 4*). **b** The TG236 marker was amplified with the primers TG236F and TG236R to generate a product of c.1,000 bp. The Cf0 allele contains a target sequence for *Ssp*I (*lane 1*) that generates fragments of 830 and 170 bp, which is absent in the Cf-ECP4 allele (*lane 2*). The Cf-ECP4/+ bulk contains both alleles (*lane 3*) and the +/+ bulk contains only the Cf0 allele (*lane 4*). Size markers are in lane '*M*'

Cf-ECP1 and Cf-ECP4 are located distal to T-DNA^{FT33} at MW

To map *Cf-ECP1* and *Cf-ECP4* more precisely we determined their location relative to the T-DNA in line FT33 (T-DNA^{FT33}), which is located c.3 cM proximal to *MW* (Rommens et al. 1992; Jones et al. 1994). The T-DNA contains a copy of the non-autonomous *Z. mays* transposon *Ds*, which carries the *E. coli uidA* (*GUS*) gene. FT33 was crossed to four Cf-ECP lines (Cf-ECP1, Cf-ECP2, Cf-ECP4 and Cf-ECP5) as the female parent and F_1 progeny were used to pollinate Cf0 plants. Test cross (TC) progeny were assayed histochemically for GUS activity and then infected with the appropriate PVX:*ECP* construct to determine their *Cf-ECP* genotype.

A χ^2 analysis confirmed that none of the four *Cf-ECP* genes assort independently of the *GUS* reporter in T-DNA^{FT33} demonstrating they are linked in repulsion (results not shown). Most progeny were either GUS-positive and exhibited wildtype symptoms of PVX infection (GUS/WT), or lacked the *GUS* reporter and exhibited PVX:*ECP*-induced necrosis (+/N). However, recombinant chromosomes were also detected (Table 2). The recombinant fraction between T-DNA^{FT33} and *Cf-ECP2* (located proximal to T-DNA^{FT33}) and T-DNA^{FT33} with *Cf-ECP5* appear consistent with previous reports (Haanstra et al. 1999, 2000).

Recombinant progeny from both crosses (GUS/N and +/WT seedlings) were used in a molecular analysis to determine their location relative to T-DNA^{FT33} (Fig. 4). FT33 × Cf-ECP4 +/WT recombinants lacked the LoxR$^{Cf\text{-}ECP4}$ allele, which is located distal to T-DNA^{FT33}, but retained TG236$^{Cf\text{-}ECP4}$, which is located proximal to it. In contrast, GUS/N recombinants lacked TG236$^{Cf\text{-}ECP4}$ but retained LoxR$^{Cf\text{-}ECP4}$ (Fig. 4). Similarly, all +/WT recombinants from the FT33 × Cf-ECP1 cross lacked the LoxR$^{Cf\text{-}ECP1}$ allele, but this was retained in GUS/N recombinants (Fig. 4). Analysis of the same recombinants with TG236 and CT116 was not possible as the Cf-ECP1 allele is not polymorphic with the *S. lycopersicum* allele, at least with the enzymes tested (results not shown). These data show *Cf-ECP1* and *Cf-ECP4* are located distal to T-DNA^{FT33} on the short arm of chromosome 1.

The genetic distance between *Cf-ECP1* and *Cf*-ECP4 with T-DNA^{FT33} (Table 2) is similar to that reported for *Cf-9* and T-DNA^{FT33} (Jones et al. 1994) suggesting they may also be located at *MW*. To confirm this, allelism tests with *Cf-9* were performed by crossing Cf-ECP1 and Cf-ECP4 lines to line M18, which contains a *Ds*-tagged allele of *Cf-9* (*Cf-9::Ds*^{M18}) that originated from T-DNA^{FT33} (Jones et al. 1994). The F_1s were used to pollinate Cf0 plants and TC progeny from both crosses were assayed for GUS (as a marker for *Cf-9*) and then infected with recombinant PVX as described above. No recombinant chromosomes were detected in 491 TC progeny from the M18 × Cf-EC4 cross, or in 572 progeny from the M18 × Cf-ECP1 cross (Table 2). These data show that *Cf-ECP1* and *Cf-ECP4* are allelic to *Cf-9* at *MW*, or very tightly linked.

Identifying T-DNA^{FT33}/ Cf-ECP recombinants for transposon tagging

Our objective is to isolate all four *Cf-ECP* genes by transposon tagging so we used marker-assisted selection to identify recombinants containing T-DNA^{FT33} *in cis* with each *Cf-ECP*. Approximately 50 GUS$^+$ TC seedlings from crosses between the Cf-ECP lines with FT33 were screened by CAPS analysis. Progeny containing *Cf-ECP1* or *Cf-ECP4* were identified using the LoxR marker (see Fig. 4); progeny containing *Cf-ECP2* or *Cf-ECP5* were identified using the markers CT116 and Cf4F3R3, respectively, which were previously shown to cosegregate with these genes (Haanstra et al. 1999, 2000).The presence of each *Cf-ECP* was confirmed by infecting self progeny with the appropriate PVX:*ECP* construct. In each case the progeny segregated at an approximate ratio of 3:1 for necrotic:wildtype individuals (results not shown).

Table 2 Linkage analysis of C*f*-ECP genes

Cross	Non-recombinant classes		Recombinant classes		Recombinant fraction
	GUS/WT	+/N	GUS/N	+/WT	
Cf0 × (FT33 × Cf-ECP4)	139	149	4	5	2.4 %
Cf0 × (FT33 × Cf-ECP1)	116	128	3	5	3.2 %
Cf0 × (FT33 × Cf-ECP5)	112	100	5	3	4.1 %
Cf0 × (FT33 × Cf-ECP2)	89	99	7	5	6.0 %
Cf0 × (M18 × Cf-ECP4)	223	268	0	0	0 (+ 0.61%)[a]
Cf0 × (M18 × Cf-ECP1)	274	298	0	0	0 (+ 0.53 %)[a]

[a] For recombination values of zero the upper limit recombination value at $P = 0.05$ for n gametes was calculated according to the formula $1-P^{1/n}$ according to Fisher and Yates (1963)

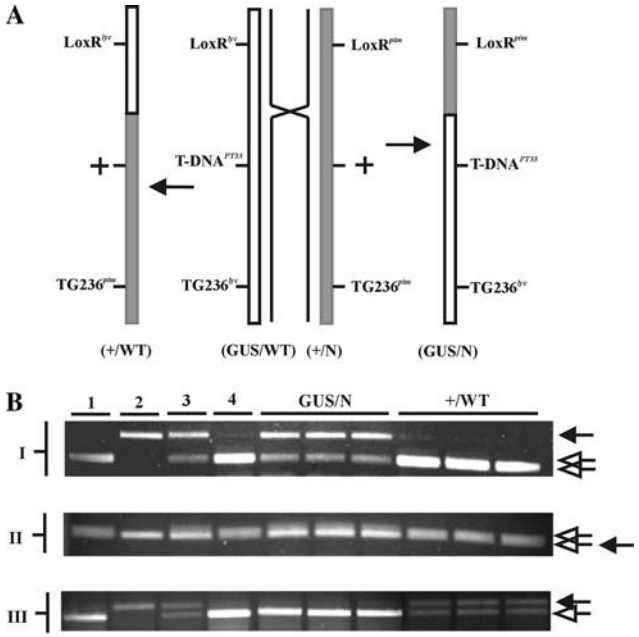


Fig. 4 Molecular analysis of recombinant TC progeny from FT33 × Cf-ECP1 and FT33 × Cf-ECP4 crosses. **a** Schematic showing molecular markers in *S. lycopersicum* (*open box*) and *S. pimpinellifolium* (*grey box*), pairing between non-sister chromatids, and the predicted haplotypes of recombinant chromosomes. **b** Molecular analysis of three GUS/N and three +/WT recombinants identified in progeny from FT33 × Cf-ECP1 and FT33 × Cf-ECP4 testcrosses using LoxR and TG236 CAPS markers. *Panel I* shows *Hinc*II digestion products of the LoxR marker on FT33 × Cf-ECP1 recombinants; *panels II* and *III* *Hinc*II digestion of LoxR and *Ssp*I digestion of TG236 from FT33 × Cf-ECP4 recombinants, respectively. Cf0 alleles are indicated with *open arrowheads* and Cf-ECP alleles with *filled arrowheads*. *Hinc*II digestion of the Cf0 LoxR allele generates two fragments of 262 and 234 bp, whereas digestion of the Cf-ECP4 allele generates a single product of 234 bp due to a short deletion within LoxR. Heterozygotes can be distinguished from Cf0 homozygotes based on the relative intensities of the 262 and 234 bp fragments (see *lanes 3* and *4* in *panel II*). *Lane 1*, Cf0, *lane 2* Cf-ECP parent, *lane 3* Cf-ECP/+ bulk, *Lane 4* +/+ bulk, and *lanes* corresponding to GUS/N and +/WT recombinants as indicated

DNA gel blot analysis of bulked segregant pools identifies candidate Hcr9s for Cf-ECP1 and Cf-ECP4

Having shown *Cf-ECP1* and *Cf-ECP4* are located at *MW* we identified cosegregating *Hcr9*s as candidate *Cf-ECP*s. DNA gel blots of Cf0 and Cf-ECP lines, together with the bulked segregant pools described above (Fig. 5) were hybridised with a *Cf-9* 5′ probe PCR-amplified from pCD-NAL9 (Jones et al. 1994). Analysis of *Bgl*II-digested DNA identified several polymorphic *Hcr9*s that cosegregate with *Cf-ECP1*, and with *Cf-ECP4* (Fig. 5).

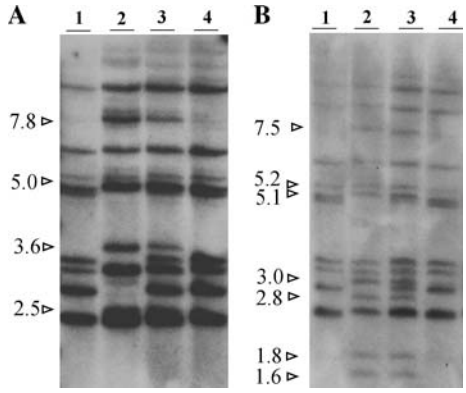


Fig. 5 DNA gel blot analysis of $F_2$1E and $F_2$4E bulked segregant pools using a *Cf-9* probe identifies candidate *Cf-ECP1* and *Cf-ECP4* genes. DNA from Cf0, Cf-ECP1, Cf-ECP4 and bulked segregant pools were digested with *Bgl*II, electrophoresed on 0.8% w/v agarose gels, and blotted as described in methods. **a** The gel blot includes *Bgl*II-digested DNA from Cf0 (*lane 1*), Cf-ECP1 (*lane 2*), the Cf-ECP1/+ bulk (*lane 3*) and the +/+ bulk (*lane 4*). **b** *Bgl*II-digested DNA from Cf0 (*lane 1*), Cf-ECP4 (*lane 2*), the Cf-ECP4/+ bulk (*lane 3*) and the +/+ bulk (*lane 4*). In both panels polymorphic *Hcr9*s that cosegregate with the corresponding *Cf-ECP* gene are indicated with *open arrowheads* and their approximate sizes are indicated in kbp

Discussion

Durable resistance to the leaf mould pathogen *C. fulvum* has been a major objective for tomato breeders (Stevens and Rick 1988; Rivas and Thomas 2005). Successive introgressions of *Cf* genes into *S. lycopersicum* provided a wealth of genetic resources that formed the basis for informative studies on *Cf* gene organisation and evolution (Jones et al. 1994; Parniske et al. 1997; Thomas et al. 1997; Kruijt et al. 2004), and Cf protein recognition specificity (Van Der Hoorn et al. 2001a; Wulff et al. 2001). Functional *Cf* genes from several *Solanum* species have been mapped to chromosome 6 (Jones et al. 1993; Dixon et al. 1996) and chromosome 11 (EA Anderson and CM Thomas, in preparation), but most *Cf* genes and *Hcr9* loci have been mapped to loci on the short arm of chromosome 1 including *MW*, *Southern Cross* (*SC*), *Northern Lights* (*NL*), *OR* and *AU* (Jones et al. 1993; Parniske and Jones 1999; Parniske et al. 1999; Haanstra et al. 1999, 2000; Yuan et al. 2002). Several studies suggest that *Hcr9* amplification and the duplication of these loci occurred prior to speciation (Parniske and Jones 1999; Parniske et al. 1999; De Kock et al. 2005).

To learn more about functional *Cf* gene loci genetic strategies have been used to identify novel genes on the short arm of tomato chromosome 1 (Haanstra et al. 2000), and genes that induce an HR upon recognition of specific *C. fulvum* ECPs, like the *S. pimpinellifolium* *Cf-ECP1*, *Cf-ECP2*, *Cf-ECP3*, *Cf-ECP4* and *Cf-ECP5* genes (Lauge

et al. 1998, 2000). The chromosomal locations of *Cf-ECP1* and *Cf-ECP4* were previously unknown and we demonstrate both genes are located distal to T-DNA^{FT33} at *MW* (Fig. 4 ; Table 2). The isolation and characterisation of both genes will increase our understanding of the molecular basis for *Cf* gene evolution at *MW*. *Cf-9* and *Cf-4* were originally introgressed from *S. pimpinellifolium* and *S. hirsutum* but they apparently originated prior to speciation and are present in many wild Solanum accessions (Kruijt et al. 2005). These two haplotypes are the most extensively characterized at the molecular level and these analyses form the basis for our current understanding of *Cf* gene evolution (Jones et al. 1994; Parniske et al. 1997 Thomas et al. 1997; Kruijt et al. 2004). Each haplotype is comprised of tandemly duplicated *Hcr9*s and *Cf* gene variation is generated by sequence exchange between *Hcr9* paralogues, and non-synonymous nucleotide substitutions in sequences encoding the solvent-exposed residues of a conserved LRR structural motif, which determine recognition specificity (Parniske et al. 1997; Thomas et al. 1997; Van Der Hoorn et al. 2001a; Wulff et al. 2001).

Previous mapping studies showed *Cf-ECP2* and *Cf-ECP3* are located at *OR* c.10 cM proximal to *MW* (Fig. 6) and together they define another complex locus for *C. fulvum* resistance (Haanstra et al. 1999; Yuan et al. 2002) while *Cf-ECP5* was mapped 3 cM proximal to *MW* at *AU* (Haanstra et al. 2000), and these reports are consistent with our test cross analyses (Table 2). The demonstration that *Cf-ECP1* and *Cf-ECP4* are located at *MW* increases the number of distinct *Cf* recognition specificities at this locus to seven (Fig. 6) and emphasises its importance in generating *Cf* gene diversity. This is particularly true in *S. pimpinellifolium* where *Cf-9, 9DC1, 9DC2*, and *9DC3* (which all induce an Avr9-dependent HR) and *Hcr9-9B* originated (Stevens and Rick 1988; Jones et al. 1994; Parniske et al. 1997; Kruijt et al. 2004).

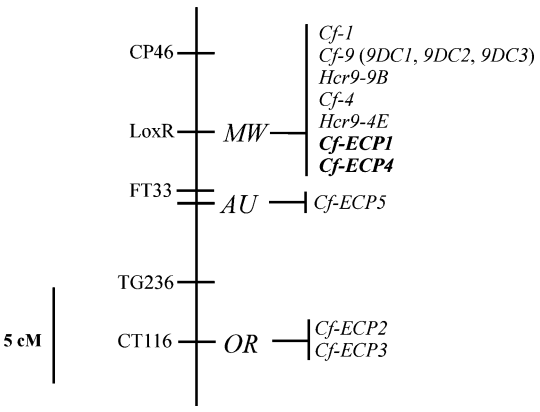


Fig. 6 A genetic map of the short arm of chromosome 1 showing the locations of ten distinct recognition specificities at the *MW*, *AU* and *OR* loci

It is assumed *Cf-ECP* genes also encode Hcr9 proteins and molecular analysis of populations where *Cf-ECP2* or *Cf-ECP5* segregated did identify candidate *Hcr9*s (Haanstra et al. 2000; De Kock et al. 2005). However, this strategy failed to identify an *Hcr9* that could induce an ECP2-dependent HR (De Kock et al. 2005). Significantly, the *Cf-ECP2* locus has not been delimited with flanking markers and it is possible that not all candidate *Hcr9*s have been tested (De Kock et al. 2005). Our analysis of bulked segregant pools identified at least four polymorphic *Hcr9*s that cosegregate with *Cf-ECP1* (Fig. 5a) and six that cosegregate with *Cf-ECP4* (Fig. 5b) so it appears the *MW* haplotypes in *S. pimpinellifolium* LA1547 (Cf-ECP1) and LA1683 (Cf-ECP4) are also comprised of duplicated *Hcr9*s. Candidate *Hcr9*s could now be tested by transient expression in *N. tabacum* or *S. lycopersicum* together with the cognate *ECP*.

An alternative strategy to cloning *Cf-ECP*s is transposon tagging using the genetically linked *Ds* element in line FT33 (Rommens et al. 1992; Fig. 6), as in the isolation of *Cf-9* (Jones et al. 1994). We identified recombinants containing each *Cf-ECP in cis* with T-DNA^{FT33} and these will be crossed to a line expressing the *Activator* transposase to induce *Ds* transposition (Jones et al. 1994). Identification of *Ds*-tagged mutants of *Cf-ECP2*, *Cf-ECP4* and *Cf-ECP5* will be achieved by testcrossing tagging parents to the appropriate ECP line to identify suppressors of the seedling lethal phenotype (Fig. 2). The frequency of *Ds*-tagged mutants of *Cf-ECP1*, *Cf-ECP4* and *Cf-ECP5* should be similar to that reported for *Cf-9* i.e., c.1 in 2.5×10^3 (Jones et al. 1994), but less frequent for *Cf-ECP2*, which is located further from FT33 (Haanstra et al. 1999). This assumes each line contains a single copy of each *Cf-ECP* but in some haplotypes, such as the *S. pimpinellifolium Cf-2* locus on chromosome 6 and the *MW* locus in LA1301, multiple copies of the functional genes have arisen through sequence duplications (Dixon et al. 1996; Van Der Hoorn et al. 2001b; Kruijt et al. 2004).

No lines expressing ECP1 were obtained in this study (Table 1), which might reflect the limited number of transformants analysed, or toxicty associated with constitutive expression of ECP1 *in planta*. Despite this, *Ds*-tagged mutants of *Cf-ECP1* could be identified in a high-throughput screen based upon suppression of PVX:*ECP1*-induced necrosis (Fig. 2). The availability of stable transgenic lines expressing ECP2, ECP4 or ECP5 and PVX-mediated transient expression of all four ECPs will prove useful for the functional analysis of cloned *Cf-ECP*s (Fig. 2). Expression of *C. fulvum* AVRs and ECPs in tomato and tobacco obviates the requirement for *C. fulvum* infections and has proved invaluable for structure-function studies of Cf-9 and Cf-4 (Van Der Hoorn et al. 2001a; Wulff et al. 2001), functional analysis of AVRs and ECPs (Kooman-Gersmann

et al. 1997; Luderer et al. 2002), and identifying novel *Cf* genes (Lauge et al. 1998, 2000).

The actual number of *Cf* gene loci in *Solanum* species has yet to be determined. Duplicated *Hcr9*s are present at *SC* and *NL* (Parniske et al. 1999; Parniske and Jones 1999) but some are pseudogenes and functional genes have yet to be identified at these loci. Only a limited analysis of *S. pimpinellifolium* accessions were required to identify *Cf-ECP* genes (Lauge et al. 1998, 2000), and more extensive analyses have shown that functional variants of *Cf-9* and *Cf-4* are widely distributed in *Solanum* species (Kruijt et al. 2005). This demonstrates that wild Solanum germplasm is an important source of *Cf* gene variation, which could be exploited for breeding, and for evolutionary analyses. Our results further highlight the importance of the *MW* locus in generating *Cf* gene novelty. Effective use of the genetic resources described here will make the isolation of all four *Cf-ECP*s by sequence homology or transposon tagging a realistic possibility. The molecular characterisation of all four *Cf-ECP*s should increase our understanding of the molecular mechanisms underlying *Cf* gene evolution at *MW*, and the molecular basis for Cf protein recognition specificity.

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