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

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Summary

Leaf mould disease in tomato is caused by the biotrophic fungus *Cladosporium fulvum*. An *Ac/Ds* targeted transposon tagging strategy was used to isolate the gene conferring resistance to race 5 of *C. fulvum*, a strain expressing the avirulence gene *Avr4*. An infection assay of 2-week-old seedlings yielded five susceptible mutants, of which two had a *Ds* element integrated in the same gene at different positions. This gene, member of a gene family, showed high sequence homology to the *C. fulvum* resistance genes *Cf-9* and *Cf-2*. The gene is predicted to encode an extracellular transmembrane protein containing a divided domain of 25 leucine-rich repeats. Three mutants exhibited a genomic deletion covering most of the *Lycopersicon hirsutum* introgressed segment, including the *Cf-4* locus. Southern blot analysis revealed that this deletion includes the tagged gene and five homologous sequences. To test whether the tagged gene confers resistance to *C. fulvum* via *Avr4* recognition, the *Avr4* gene was expressed *in planta*. Surprisingly, expression of the *Avr4* gene still triggered a specific necrotic response in the transposon-tagged plants, indicating that the tagged resistance gene is not, or is not the only gene, involved in *Avr4* recognition. Mutants harbouring the genomic deletion did not show this *Avr4*-specific response. The deleted segment apparently contains, in addition to the tagged gene, one or more other genes, which play a role in the *Avr4* responses. The tagged gene is present at the *Cf-4* locus, but it does not necessarily recognize *Avr4* and is therefore designated *Cf-4A*.

Introduction

Cladosporium fulvum is a biotrophic fungal pathogen causing leaf mould on its host tomato (*Lycopersicon esculentum*). *Cladosporium fulvum* enters the plant via the leaf stomata and colonizes the intercellular spaces in susceptible tomato genotypes (Van den Ackerveken *et al.*, 1994). During sporulation, the fungus emerges from the stomata, preventing them from closing, causing wilting of the leaves. In a resistant tomato genotype the infection process is blocked at an early stage due to recognition of the pathogen and subsequent activation of host defence responses.

The gene-for-gene hypothesis (Flor, 1942) proposes that host resistance is based on the recognition of the fungal avirulence (*Avr*) gene product (or elicitor) by the matching host resistance (*R*) gene product. Recognition results in an incompatible interaction. The different strains of *C. fulvum* are classified in various races on the basis of their ability to overcome the various monogenic resistance genes present in different tomato genotypes. Two avirulence genes of *C. fulvum*, *Avr4* (Joosten *et al.*, 1994) and *Avr9* (Van den Ackerveken and De Wit, 1992), have been cloned and characterized. Both encode small cysteine-rich proteins, which are secreted by the fungus in the apoplast, but their precise role in the infection process is as yet unclear.

A number of *R* genes conferring resistance to different races of *C. fulvum* have been introgressed from wild species into the cultivated tomato. Some examples are *Cf-2* and *Cf-9* from *L. pimpinellifolium*, *Cf-5* from the land race *L. esculentum* var. *cerasiforme* and *Cf-4* from *L. hirsutum*. On the basis of the gene-for-gene model, these *R* genes were suggested to encode receptors specific for the corresponding *Avr* product. By selection for the gene involved in the hypersensitive response (HR) to specific elicitors, the corresponding *R* gene might be cloned. This strategy was applied to clone the *Cf-9* resistance gene (Jones *et al.*, 1994). A transposon tagging population of plants expressing both the *Cf-9* and the *Avr9* gene was screened for survivors. The surviving mutants, no longer showing a systemic HR, had a transposon tag in *Cf-9*. This method of selection only gives the gene products specifically recognizing and responding to *Avr9*. This gene is not necessarily the only resistance gene present that recognizes race 9 of *C. fulvum*.

Alternatively, with a positional cloning strategy, resistance genes can be isolated (for a recent *R* gene review see Bent, 1996). Candidate *R* genes isolated in this way can subsequently be tested for complementation of susceptible

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plants using an inoculation screen with the pathogen of interest. The *Cf-2* locus of tomato, which was found to contain two functional resistance genes, was isolated with this approach (Dixon *et al.*, 1996). Both genes confer resistance to *C. fulvum*; however, since the Avr2 elicitor has not been isolated no data are available regarding whether these two *Cf-2* resistance genes interact with the Avr2 elicitor. A disadvantage of this method is that the transgene might have different characteristics (e.g. expression level) than the endogenous gene. Furthermore, it is never certain if the genes that complement are the (only) ones giving *in planta* resistance.

To isolate the *Cf-4* gene that confers *in planta* resistance to *C. fulvum* and to overcome the limitations of the above methods, we used a targeted transposon tagging approach. The heterologous *Ac/Ds* system from maize was used to inactivate the *in planta* resistance gene by integration of the transposable *Ds* element. Subsequent loss of disease resistance in the mutated progeny was assayed with an inoculation assay using a *C. fulvum* race 5 that expresses the Avr4 elicitor (Joosten *et al.*, 1994). Since the *Avr4* gene has been cloned, the plants with a tagged *C. fulvum* resistance gene could be tested for their response to the elicitor.

Five plants susceptible to infection with race 5 of *C. fulvum* were obtained from the tagging population. Two plants showed an unstable phenotype in the presence of the transposase, suggesting a transposon-induced mutation. Isolation of the tagged gene showed that it has high sequence homology to *Cf-9* and *Cf-2*. These genes all encode proteins with a leucine-rich repeat (LRR) domain and a region that is expected to anchor the protein to the cell membrane.

Surprisingly, these transposon-tagged plants still show a specific response to Avr4, indicating that these susceptible plants still recognize the Avr4 elicitor produced by the fungus. Because the tagged gene present at the *Cf-4* locus does not necessarily recognize Avr4, the gene has been designated *Cf-4A*.

Results

Targeted tagging experiment

The two-component *Ac/Ds* system was used to isolate the gene conferring resistance to race 5 of *C. fulvum*. The tagging population of heterozygous MM-Cf4 plants (see the Experimental procedures) was screened for susceptibility to infection by race 5 of *C. fulvum*. Twenty thousand 2-week-old seedlings were inoculated with conidiospores of the fungus (see the Experimental procedures). Five plants, derived from independent parents, showed yellowing and subsequent abscission of the cotyledons. In a later stage of infection sporulation of the fungus at the lower side of

Table 1. Genotypes and phenotypes of plants described in the text

Plant	Genotype	Race 5 <i>C. fulvum</i>	PVX::Avr4	Note
MM-Cf0	<i>Cf-0/Cf-0</i>	S	–	Wild type
MM-Cf4	<i>Cf-4/Cf-4</i>	R	+	Wild type
4	<i>Cf-4/Cf-0</i>	S*	+ (delay)	Transposon tag
65	<i>Cf-4/Cf-0</i>	S*	+ (delay)	Transposon tag
17	<i>Cf-4/Cf-0</i>	S	–	Deletion mutant
47	<i>Cf-4/Cf-0</i>	S	–	Deletion mutant
84	<i>Cf-4/Cf-0</i>	S	–	Deletion mutant

MM-Cf0 and MM-Cf4 are the parents used for generating the tagging population. The numbers indicate the susceptible mutants obtained from the tagging experiment. The column headed Race 5 *C. fulvum* indicates resistance (R) or susceptibility (S) of the plants upon inoculation of the fungus. An asterisk indicates that susceptibility of these mutants changes during development. The PVX::Avr4 column indicates the absence (–) or presence (+) of necrosis upon infection of the virus.

the leaves indicated that loss of resistance had occurred. An infection assay of progeny derived from these mutants using two different *C. fulvum* isolates of race 5, confirmed the susceptibility of these five mutants to the fungus (Table 1). The presence of a functional *Avr4* gene in both *C. fulvum* strains was confirmed in two ways: first by sequence analysis, and second via injection of apoplastic fluid (isolated from compatible interactions involving both races) containing the elicitor in a MM-Cf4 plant. (see the Experimental procedures).

To examine whether loss of resistance was transposon-induced, a screen for revertants was performed. To this end, the transposed *Ds* was reactivated by *Ac*. Mutants without the transposase were crossed to transgenic MM-Cf0 plants expressing the transposase, while the mutants with the transposase were self-pollinated. In the progeny of two of the five mutants, reversion from susceptible to resistant was observed at a frequency of 2–3% of the 250–500 plants tested, suggesting that the mutation is transposon-induced.

A test cross of the susceptible mutants to MM-Cf4 plants revealed that the complete progeny was resistant to infection by race 5 of *C. fulvum*, implying that these mutations are recessive.

The above infection assays were always performed on 2-week-old seedlings. No differences in the density of sporulation of the fungus on the leaves were observed between the five mutants and the susceptible MM-Cf0 control plants. In order to identify whether disease susceptibility is constant during plant development, homozygous progeny of the mutants was inoculated 4 and 8 weeks after germination. The degree of susceptibility of the three stable mutants did not change during development compared to the susceptible MM-Cf0 control plants. The two unstable

Figure 1. Western blot (immunoblot) analysis of 100 µl apoplastic washing fluid (AF) isolated from infected plant material 3 weeks post-inoculation. The AF was electrophorized on a denaturing 15% SDS-PAGE, blotted and subsequently probed with the ECP2 polyclonal antibody. Four-week-old plants were infected with race 5 of *C. fulvum*. Cf0 and Cf4 are the susceptible and resistant parental lines. The numbers represent the susceptible mutants, 4 and 65 show an unstable phenotype, while 17, 47 and 84 are stable mutants. The sizes of the molecular weight markers are indicated in kilodaltons (kDa).

mutants, however, showed a decreasing susceptibility with time. Infection at 4 weeks led to diseased plants with repressed sporulation of the fungus on the leaves, while at 8 weeks no sporulation at all could be detected. To determine whether this decreased sporulation (in 4-week-old plants) coincides with a lower biomass of fungus in the leaves, the amount of the *C. fulvum* infection-specific, extracellular protein ECP2 (Wubben *et al.*, 1994) was quantified (Figure 1). In both unstable mutants (numbers 4 and 65) the amount of ECP2 was decreased compared to the MM-Cf0 and the stable mutants (17, 47 and 84) that were inoculated with the same strain. In the resistant MM-Cf4 plant no ECP2 was detectable.

In summary, screening a transposon tagging population yielded five susceptible mutants; two with an unstable phenotype and three with a stable phenotype in the presence of the *Ac* transposase. At a 2-week-old stage all mutants were equally susceptible to infection by race 5 of *C. fulvum*; however, the susceptibility of the unstable mutants changed during development.

Molecular characterization of the *Ds*-tagged mutants

Southern blot analysis of the two plants segregating for the unstable phenotype in the progeny (plants 4 and 65), using an internal *Ds* probe, showed that in these plants the *Ds* element was excised from its original position and had integrated at a new position (data not shown).

The *Ds*-flanking sequences were isolated by plasmid rescue and anchored PCR (see the Experimental procedures). Southern blot analysis of *Bgl*II-digested DNA of different near-isogenic lines (MM-Cf9, MM-Cf4 and MM-Cf0), and plant 4 probed with a *Ds*-flanking sequence (510 bp 3'-flanking sequence of plant 4; see the Experimental procedures), showed that the *Ds* element integrated in a member of a family of homologous sequences (Figure 2). Figure 2 shows that insertion of the 7.2 kb *Ds* element led to loss of a 1.8 kb *Bgl*II fragment and formation of a novel 9 kb fragment. Similar results were obtained for plant 65 (data not shown); in this case the *Ds* element had

Figure 2. Southern blot analysis of the near-isogenic lines and of one of the transposon-tagged mutants (plant 4).

Plant DNA was digested with *Bgl*II and after electrophoresis blotted to HybondN⁺ membranes and hybridized with the 3'-*Ds* flanking sequence obtained via plasmid rescue (see the Experimental procedures). Sizes of the fragments are indicated on basis of the sizes of lambda DNA digested with *Hind*III/*Eco*RI. The arrow indicates the position of the 1.8 kb fragment that is absent in the tagged mutant (4).

inserted in a 0.8 kb *Bgl*II fragment to which the previous probe did not hybridize. Southern blot analysis showed that in both plants the transposon integrated in the same 1.0 kb *Hind*III fragment (data not shown). These data indicate that the two unstable mutants both have a *Ds* insertion at different positions in the same restriction fragment. Furthermore, the *Ds* elements have integrated in one of the members of a group of homologous sequences.

Molecular characterization of the stable mutants

As mentioned above, MM-Cf0 and MM-Cf4 plants show a number of specific bands detected on Southern blots using probes derived from the *Ds* flanking sequences. Figure 3 shows an autoradiogram of a Southern blot containing *Bgl*II-digested plant DNA probed with the 3'-*Ds* flanking sequence of plant 4. In MM-Cf0 plants eight bands were identified after high stringency washes (see the Experimental procedures), while in MM-Cf4 and MM-Cf9 plants 12 bands are visible. Six bands co-migrated and could be identical in these isogenic lines. Plant 65 showed the normal RFLP pattern of a heterozygote, because the *Ds* element integrated in a different *Bgl*II fragment to which the probe did hybridize. Southern analysis of the three susceptible stable mutants (numbers 17, 47 and 84) showed that the homologous bands specific for a MM-Cf4 plant were absent, including the tagged 1.8 kbp band absent in

Figure 3. Southern blot analysis of tomato DNA. DNA of the different near isogenic lines (MM-Cf0, MM-Cf4 and MM-Cf9), one of the transposon tagged mutants (65) and the three stable mutants (17, 47 and 84) was digested with *Bgl*II. The blot was hybridized with a probe derived from the 3'-*Ds* flanking sequence of plant 4 (510 bp PCR product Cf4r1-Lds10) and washed at high stringency. Lanes M contain lambda DNA digested with *Eco*RI/*Hind*III as size markers, sizes in kilobases are indicated at the right. Cf-4- and Cf-0- numbers indicate hybridizing sequences specific for the MM-Cf4 and MM-Cf0 isolines, respectively.

Figure 4. Detail of the genetic map representing the short arm of chromosome 1 of tomato around the *Cf*-4 locus. The numbers indicate the RFLP markers that have been used to map the introgressed *L. hirsutum* DNA (vertical bar) containing the *Cf*-4 locus. Map data are according to Balint-Kurti *et al.* (1995)

plant 4 (Figure 2). In plants 17 and 47 one band specific for MM-Cf0 was also missing (band Cf-0-2; Figure 3).

In previous studies, the *Cf*-4 resistance gene has been mapped between the markers CP46 and TG236 (Figure 4)

(Balint-Kurti *et al.*, 1994; Bonnema *et al.*, 1997). The *L. hirsutum*-introgressed region in *L. esculentum* cv. MoneyMaker covers the markers CT233 and CP46 (Balint-Kurti *et al.*, 1994). RFLP analysis (see the Experimental procedures and Table 2) of the three stable mutants revealed that they still contained the introgressed *L. hirsutum* pattern for CT233 (the most distal marker). The other markers present in the introgressed region (CP46, LRRf1r1 and TG301), however, no longer detected this *L. hirsutum* DNA, indicating the loss of this region. The genetic distance of CT233 to TG236, mapped in an interspecific *L. esculentum* × *L. pennellii* cross, covered maximally 13 cm (Balint-Kurti *et al.*, 1995). These data indicate that in the three independent stable mutants a genomic deletion had occurred of part of the introgressed *L. hirsutum* DNA segments, including the *Cf*-4 locus.

Thus, the three independent stable mutants do not have transposon insertions, instead they have a genomic deletion covering the *Cf*-4 locus.

Cloning and sequencing of the *Cf*-4A gene

The *Ds* element used for tagging was suitable for plasmid rescue. Using a *Bgl*II digest, the 1.8 kb *Ds* flanking DNA sequences of plant 4 were isolated. To isolate the flanking sequences of plant 65, an anchored PCR approach was used. A fragment of 0.8 kb, 3'-*Ds* flanking, was isolated using *Bcl*I and a 0.7 kb 5'-flanking fragment was isolated using *Bgl*II. RT-PCR was used to isolate the 3'-end of the gene (see the Experimental procedures). The sequence data were confirmed by sequencing a lambda (λGem11) clone containing the *Cf*-4A gene isolated from a genomic MM-Cf4 library.

The flanking sequences showed that both *Ds* elements had integrated at different positions in the same ORF (Figure 5). The ORF of the tagged gene spanned 2565 bp (accession number Y12640), encoding a protein of 855 amino acids. The integration sites of the two *Ds* elements are indicated in Figure 6 by a capital V. Comparison of the DNA sequence of this ORF to the sequences in Genbank via BlastN (Altschul *et al.*, 1990) revealed it had the highest homology to *Cf*-9 (Jones *et al.*, 1994), which was 91% identical. Both *Cf*-2 genes (Dixon *et al.*, 1996) also had a high homology to the tagged gene (see the Experimental procedures for BlastN data). Comparing the deduced protein sequence to sequences in Genbank using BlastP (Altschul *et al.*, 1990) also gave the highest homology to the *Cf*-9 and *Cf*-2 proteins.

The most striking features of the protein encoded by the tagged gene were its 22 leucine-rich repeats (LRR) present in domain III, spaced by 66 amino acids from three additional LRR (domain V). Domains II to VI showed homology to polygalacturonase-inhibitor proteins (PGIP), while domain I encoded a putative signal peptide. Domain VII

Table 2. CAPS markers tested on the stable deletion mutants

CAPS	Primer	Length	Enzyme
TG51	F:GGCTATGAAATCGGTGAGTCAG R:TGAAGGCTAATGTAAGACATGAAG	1500	Taq1
TG236	F:AGCCCAAGAAAACCAACCCAAAGTG R:GGGTCGAGAGATTACATAACGTTG	1000	Ddel
CT233	F:CCCTATCATTCCACAAAACAGCA R:GGTGTCTTTCTACCTTCTGAGCCA	1650	Rsal
CP46	F:AAGTTAGGGTGAACACAATATCTG R:CTGGAAACTCATTACTTCTATTTA	1000	EcoRV
TG301	F:AGAGCTCAGGATCAACAGAACACGAA R:TTCTGCCAATTTCTTTGACCAACC	800	Hinfl
Lrrf1r1	F:ATCCAATTCCTAACTGG R:ATAGATCGTGGGACTTTCC	540	BglII

Ds insertions in Cf-4A

Figure 5. Schematic representation of the two *Ds* insertions in the *Cf-4A* ORF. The bar indicates distances in bases. Triangles represent the *Ds* insertions in plants 4 and 65. The end of the ORF is indicated by Stop; E = *EcoRI*, B = *BglII* and H = *HindIII*.

was rich in acidic amino acid residues, while domain IX was rich in basic amino acid residues. The region in between was a hydrophobic, possibly transmembrane, region. The predicted structure with these domains was similar to that proposed for the Cf-9 (Figure 6) and Cf-2 proteins (Dixon *et al.*, 1996).

Alignment of the deduced protein sequences of Cf-9 and Cf-4A showed that both proteins are 84% identical and over 90% similar to each other (Figure 6). The Cf-4A protein was expected to have a structure very similar to Cf-9 except for a small 10 amino acid deletion at position 57–66 and a three amino acid insertion at position 350. Most differences were found in the first 600 amino acids of the gene, which were 83% identical, while the last 256 amino acids were conserved (91% identical) and most differences were conserved changes (97% similarity).

Characterization of the tagged gene

To study whether the tagged gene, conferring resistance to Avr4-expressing races of *C. fulvum*, interacts with the Avr4 elicitor, the response of the mutants to the Avr4 elicitor was examined. Apoplastic fluid (AF) from a compatible interaction between race 5 of *C. fulvum* and a MM-Cf0 tomato was isolated. This AF, which contains the Avr4 elicitor, was injected in two different leaflets of MM-Cf0 and MM-Cf4 plants and each of the five susceptible

mutants. Wild-type MM-Cf4 plants gave local necrosis on the site of injection, while MM-Cf0 plants did not, though chlorosis was occasionally observed. The three stable mutants did not exhibit any necrotic response upon injection of the AF, while plants 4 and 65 unexpectedly did show necrosis (data not shown).

To assay the effect of Avr4 in more detail, the *Avr4* gene was expressed *in planta* using an expression system based on potato virus X (PVX) (Chapman *et al.*, 1992). The Avr4 protein was targeted to the apoplast by the PR-1A signal sequence of *Nicotiana tabacum* (Joosten *et al.*, 1997), as in the PVX::Avr9 vector used by Hammond-Kosack *et al.* (1995). Single cotyledons of 10–14-day-old seedlings were inoculated with PVX::Avr4. MM-Cf0 tomato plants did not show a specific response upon systemic infection by PVX::Avr4, except for some mild mosaic virus symptoms at about 10 days after inoculation, similarly as described previously (Joosten *et al.*, 1997). Tomato plants carrying the Cf-4 resistance locus showed necrotic spots on the cotyledons 3–4 days after inoculation. These enlarged and led to abscission of the cotyledon about 5 days after inoculation. Necrotic spots, emerging at the lower leaves of the plants, could be observed around 4–5 days after inoculation, eventually leading to whole plant death. These symptoms were similar to those described for MM-Cf9 plants inoculated with PVX::Avr9 (Hammond-Kosack *et al.*, 1995).

The progeny of the stable mutants, with a large genomic deletion containing the Cf-4 locus (plants 17, 47 and 84), were inoculated with PVX::Avr4. No necrosis was observed, but mosaic symptoms were observed about 10 days after inoculation, indicating a successful systemic infection by the Avr4-expressing virus. This proves that the gene(s) responsible for the Avr4-specific response is located in the deleted region.

Twenty progeny plants derived from the two *Ds*-tagged plants (4 and 65) were selected for homozygosity for the *Ds* insertion. Inoculation of these progeny plants with PVX::Avr4 did lead to specific necrotic responses upon

Cf-4A	MGCVKLIFFMLYVFLFQLVSSSS	23	I	Putative signal sequence
Cf-9	-D---V-L--T---AL---			
Cf-4A	LPHLCPEDQALALLQFKNMFTINPNASNYCYDR	56	II	N-terminal
Cf-9	-----S-----I			
Cf-4A	RTLWNKSTSCCSWDGVHCDETIGQ	81		
Cf-9	RTYVDIQSYPTL-----			
Cf-4A	VIEDLGCSQLQGKFSNSS	101	III	22 imperfect LRRs
Cf-9	--A--R-----			
Cf-4A	LFQLSNLKRDLSSNDFTGSPISPK	126		
Cf-9	-----F-N---L---			
Cf-4A	FGEFSDLTHLDLSDSNFTGVIPSE	150		
Cf-9	-----N-----H-S--L---			
Cf-4A	ISHLSKLHVLRIISDQYKLSLGPINFELL	178		
Cf-9	-C-----C--G--V-Y----			
	V Ds#4			
Cf-4A	LKNLTQLRELHLESVNISSTIP	200		
Cf-9	-----N-----			
Cf-4A	SNFSFHLTNLRSLYTELRGVLPER	224		
Cf-9	---S---T-Q--G---H-I----			
Cf-4A	VFHLSNLELDLSDYNFQLTVRFPPTI	250		
Cf-9	-----QS-H--V-----K			
Cf-4A	WNSSASLVKLYLSRVNIAGNIPDS	274		
Cf-9	-----MT--VDS----DR--K-			
Cf-4A	FSYLTALHELDVYTNLSGPIPKP	298		
Cf-9	--H--S---Y-GRC-----			
Cf-4A	LWNLTNIESLDLYNHLGPIQ	321		
Cf-9	-----VF-H-GD-----SH			
Cf-4A	LPIFEKLSLTGLNNMLDGGLEF	344		
Cf-9	FT-----R-S-V--F-----			
Cf-4A	LSFNRSWTQLEELDFSSNSLTGPIPSN	371		
Cf-9	-----R--L-----			
Cf-4A	VSGLRNLQSLYLSSNNLNGSIPSW	395		
Cf-9	I---Q--EC-----H-----			
Cf-4A	IFQLPSLRSLDLSNMTFSGKIQEF	419		
Cf-9	--S---VE-----			
Cf-4A	KSKTLSIVTLKQQLKGPINPS	441		
Cf-9	-----A-----K--R----			
Cf-4A	LLNQESLQFLLSHNNISGHISSS	465		
Cf-9	---KN--L-----A-----			
Cf-4A	ICNLKILMVLDLGSNNLEGTIPQCV	490		
Cf-9	-----T-IL-----			
Cf-4A	VERNEYSHLDLNRLSGTINIT	514		
Cf-9	-----K-----			
Cf-4A	FSIGNSFRAISLHGKLTGKVPRS	538		
Cf-9	--V--IL-V-----			
	V Ds#65			
Cf-4A	LINCKYLTLLDGNQINDTFPNW	562		
Cf-9	M-----M-----			
Cf-4A	LGYSQLKILSLRSNKLHGPIKSSGN	588		
Cf-9	-----F-----			
Cf-4A	TNLFMRQLQLDLSSMGFSGNLPERI	613		
Cf-9	-----G-----			
Cf-4A	LGNLQTMKKFDENTRFPEYISDRY	637	IV	spacer region
Cf-9	-----EI--S-G-----P-			
Cf-4A	-IYYDYLTTITTKQDYDSVR	657		
Cf-9	D---N-----S-----			
Cf-4A	IFTFMNIINLSKNRFEGRIPSI	679		
Cf-9	-LDS-----H-----			
Cf-4A	IGDLVGLRTINLSHNLEGHIPVS	703	V	3 imperfect LRRs
Cf-9	-----A-----			
Cf-4A	LQNLSVLESLLSSNKISGAIPQQ	727		
Cf-9	F-----E-----			
Cf-4A	LASLTFLEVINLSHNHLVGCIPKG	751		
Cf-9	-----			
Cf-4A	KQFDSFGNTSYQGNGLRGFPLSTHCGG	779	VI	Unknown function
Cf-9	-----KL---			
Cf-4A	DDQVTTPAELDQQQEEED	797	VII	Acidic region
Cf-9	E-----EE---			
Cf-4A	SPMISWQGVLMGYGCLVIGLSVIYIMWSTQYPAWFS	834	VIII	Possible membrane spanning region
Cf-9	-----V-----			
Cf-4A	RMDLKLERIITTRMKKKKKRY	855	IX	Basic anchor
Cf-9	-----H---K-----			

Figure 6. Protein alignment of the deduced amino acid sequences of the tagged Cf-4 and Cf-9 (Jones *et al.*, 1994) proteins. Identical amino acids are indicated with a bar (—), missing ones with a dot (.). The proteins are depicted according to the proposed domains. The loose consensus sequence of the leucine-rich repeats (LxxLxxLxxLxxNxxLxxIPx) is indicated in Italics. A **V** represents the *Ds* insertions found in plants 4 and 65.

infection; however, there was a 1–2 day delay before the onset of the necrotic symptoms and whole plant death compared to the PVX::Avr4-inoculated MM-Cf4 controls.

Inoculation of cuttings derived from the transposon-tagged plants confirmed that both parents also gave this necrotic response upon PVX::Avr4 infection. These data are in

agreement with those obtained from the AF injection studies described above.

A disadvantage of the above methods is that quantification of symptoms is difficult due to variation in the amount of Avr4 protein actually present in the plant. To overcome this variation the mutants were also crossed to transgenic MM-Cf0 tomato plants constitutively expressing Avr4. Twenty progeny derived from each of the mutants and MM-Cf0 and MM-Cf4 crossed to MM-Cf0::Avr4 were scored for the onset of necrotic symptoms. Nine days after sowing, the first necrotic symptoms around the midvein of the cotyledons appeared in the MM-Cf4/Avr4 seedlings; these enlarged and led to plant death after approximately 11 days. The two transposon-tagged mutants did not show symptoms up to day 11, after which the process was similar to the MM-Cf4/Avr4 seedlings, leading to whole plant death 3–4 days later. The MM-Cf0 plants and the stable mutants (17, 47 and 84) did not give necrotic symptoms. Three different seed batches were tested at different times and, although the timing of symptoms was determined by greenhouse conditions and could vary by 1–2 days, the time delay found for the tagged plants was reproducible between experiments.

In summary, the two *Ds*-tagged mutants, susceptible to *C. fulvum* race 5 infection, still showed Avr4-specific necrotic responses, but these were delayed. The three mutant plants with a deletion of the *Cf-4* locus did not show Avr4 responses.

Discussion

To clone the tomato gene conferring disease resistance to *C. fulvum*, a transposon tagging population was screened using a *C. fulvum* race 5 inoculation assay. Five susceptible plants were identified (Table 1). Two plants contained independent transposon insertions in a gene showing high sequence homology to the *Cf-9* resistance gene (Jones *et al.*, 1994). Three mutants showed a large genomic deletion covering most of the introgressed region, including the *Ds* tagged *Cf-4A* gene. While the deletion mutants exhibited the same degree of susceptibility as the susceptible MM-Cf0 plants, the susceptibility of the transposon-tagged plants changed during development.

Structure of the Cf-4A gene

The ORF of the tagged *Cf-4A* gene of 2565 bp was predicted to encode a protein of 855 amino acids. On the basis of the gene sequence, using the NetPlantGene algorithm (Hebsgaard *et al.*, 1996), no introns in the ORF were expected. This was confirmed by sequencing of products that were obtained via RT-PCR (data not shown). A small putative intron (50–80 bp) found in the 3'-untranslated region was not analysed further.

The deduced amino acid sequence of the protein encoded by the tagged gene contains LRR. This motif has also been found in a number of cloned resistance genes (for a recent overview see Bent, 1996). Similar to the two cloned *C. fulvum* resistance genes, *Cf-2* (Dixon *et al.*, 1996) and *Cf-9* (Jones *et al.*, 1994), the LRR region was divided into two regions separated by a short stretch of amino acids. The LRR are expected to play a role in protein–protein interactions (reviewed by Kobe and Deisenhofer, 1994). The predicted extracellular position of the protein (putative signal peptide and transmembrane domain) fits well with the infection process of the fungus that invades the intercellular spaces of the leaves. Moreover, both the Avr4 and Avr9 elicitors, for which the encoding genes have been cloned, were isolated from the apoplast (Joosten *et al.*, 1994; Van Kan *et al.*, 1991). This suggests that the LRR region of the *R* gene product might interact directly with avirulence products produced by the fungus. However, there is no biochemical evidence that the *Cf* gene product itself does bind the Avr product; the Avr9 elicitor also binds with a high affinity to plasma membranes isolated from leaves of a tomato genotype lacking the *Cf-9* resistance gene (Kooman-Gersman *et al.*, 1996).

Aligning the deduced protein sequences of Cf-4A and Cf-9 (Figure 6) and both Cf-2 homologues (data not shown) reveals that the first 16 LRR are the most divergent. The C-terminal part of the Cf-4A protein containing the last three LRR and the membrane-spanning region shows over 96% similarity to Cf-9. This supports the proposed model of Dixon *et al.* (1996) in which the specificity lies in the N-terminal part of the gene, while the conserved C-terminal part might be involved in the interaction with downstream components of a signalling cascade. Since both transposons were inserted in the 5'-part of the gene, a truncated protein could still be formed that lacks the C-terminal part. This truncated polypeptide is expected to be non-functional and probably is not able to interact with downstream components. Via domain swapping studies between the *Cf-4A* and *Cf-9* genes, the region(s) encoding specificity to race 5 of *C. fulvum* might be localized. Southern blot analysis also indicated that the 5'-region of this gene family is variable while the 3'-end is conserved. Probes derived from the 5'-part of the *Cf-4A* gene show 8–12 hybridizing bands at high stringency washing while probes derived from the 3'-end show 20–30 bands (data not shown).

Analysis of mutants with a genomic deletion

The cause of the deletions in the three susceptible mutants is not known. Since the parents used to produce the tagging population were heterozygous for the closely linked *Ds* element (*Ft33*), only half of the progeny inherited the *Ds* element. The three plants with the deletion did not carry the *Ds*-containing T-DNA (data not shown) so the deletion

is not transposon induced. However, instability of loci containing homologous sequences, like the ones containing resistance genes, has been reported before (Bennetzen *et al.*, 1988; recently reviewed by Crute and Pink, 1996). For example, the *Rp* genes of maize that confer resistance to the fungus *Puccinia sorghi* have a high frequency of meiotic instability (Pryor, 1987). Molecular analysis showed that the instability is mainly caused by unequal crossing-over at meiosis (Sudapak *et al.*, 1993). In another study, a similar example of unequal exchange between LRR encoding sequences within and between adjacent resistance genes in the *M* cluster of Flax has been observed (Anderson *et al.*, 1997). A prerequisite for these phenomena is clustered, homologous sequences, which could pair intergenically. For the *Cf-4* locus the unequal cross-over model could explain the observed deletion mutants. Use of probes derived from the tagged gene showed that other homologous sequences are present in the introgressed region. The observation that in two out of the three deletions a *Cf-4* homologous sequence outside the introgressed region was also missing, fits with the observation of intergenic recombination with '*Cf-4*' homologous sequences, mapping proximal or distal to the introgressed *Cf-4* *L. hirsutum* cluster. The presence of these clusters proximal and distal to *Cf-4* was indeed observed by Bonnema *et al.* (1997) and Parniske *et al.* (1997). Also, the relatively high frequency by which these mutants were found (3 in 20 000; 1.5×10^{-4}) is comparable with the reported frequencies for the *Rp1-J* (5.1×10^{-4}) and *Rp1-G* (3.2×10^{-3}) resistance genes (Sudapak *et al.*, 1993). The high mutation rate observed for these resistance loci and the *Cf-4* locus indicates the dynamic behaviour of these regions. The clustered homologous sequences at these loci might be employed by the plant to generate diversity and new resistance specificities (Bent, 1996; reviewed by Crute and Pink, 1996; Richter *et al.*, 1995). It would be interesting to test the plants, with the deletion of the *Cf-4*-specific homologous sequences, using different races of *C. fulvum* or different pathogens in order to identify whether any other resistance specificity is also lacking.

Function of the *Cf-4A* gene

Inactivation of the *Cf-4A* gene by a transposon insertion, or deletion of the region containing this gene, leads to susceptibility to infection by *C. fulvum* race 5, a strain highly expressing the *Avr4* gene (Joosten *et al.*, 1997). To examine the role of the tagged gene in *Avr4* recognition, the elicitor was injected in the leaves or the gene expressed *in planta* via the PVX expression system. The susceptible mutants having a deletion of most of the introgressed *L. hirsutum* region (covering the *Cf-4* locus) no longer responded to the *Avr4* elicitor. Unexpectedly, however, the transposon-tagged plants (4 and 65) did respond, both

upon injection of the elicitor and upon expression of the *Avr4* gene *in planta* either by PVX::*Avr4* or as a transgene. This specific necrotic response to the elicitor was delayed compared to MM-*Cf4* plants. These data indicate that the tagged gene giving resistance to race 5 of *C. fulvum* is not the gene, or not the only gene, in tomato causing recognition and response to *Avr4*. The (other) gene(s) responsible for the response to *Avr4* must be located in the region absent in the deletion mutants. One of these homologous genes (*Cf-4*) mapping at this position has been cloned (Parniske *et al.*, 1997; Thomas *et al.*, 1997) and was indeed shown to confer resistance through *Avr4* recognition in transgenic plants. The *Avr4*-specific response due to the *Cf-4* gene product is not sufficient to confer fungal resistance at early stages of development of the two transposon-tagged plants. Currently we are investigating the expression pattern of the *Cf-4* gene during development to examine if it might account for the decreased susceptibility of the transposon-tagged mutants during later stages of development. The *Cf-4* locus could function similarly as the *Cf-9* locus (Parniske *et al.*, 1997). At least two *Cf-9* homologous genes present at the *Cf-9* locus confer in transgenic plants (at least partial) disease resistance to the same race 5.9 of *C. fulvum* as the *Cf-9* gene, but with a different specificity and at different developmental stages.

These findings suggest the presence of two different *R* genes at the *Cf-4* locus both involved in resistance to race 5 of *C. fulvum*, similar to the situation at the *Cf-2* locus (Dixon *et al.*, 1996) and *Cf-9* (Parniske *et al.*, 1997). A transposon insertion in one of the genes is not expected to lead to susceptibility, unless there is crosstalk between these genes. The phenomenon that homologous sequences/genes influence each other's expression via various mechanisms, for instance sense or antisense suppression, has been reported (reviewed by Stam *et al.*, 1997). The *Ds* element used (*Ft33*) contains promoter-like sequences in one of its borders that can lead to transcription (sense or antisense) of flanking sequences (Rudenko *et al.*, 1994). If the suppression of *Cf-4* is caused by the transposon insertion in *Cf-4A* no correct restoration of the reading frame of the tagged gene is necessary to obtain resistant progeny. This has indeed been observed; footprint analysis of resistant revertants, having a normal response to *Avr4* (data not shown) derived from plants 4 and 65, revealed that some plants show footprints leading to a shift in reading frame (Takken *et al.*, in preparation).

It is not known whether the *Cf-4A* gene product is involved in recognition of *Avr4* or a different *Avr* product. Joosten *et al.* (1997), showed that virulence of *C. fulvum* races on MM-*Cf4* plants is always correlated with a mutation in the *Avr4* gene. This implies that *Avr4* is a major determinant for induction of resistance. However, these natural isolates were identified on the basis of their ability

to infect MM-Cf4, so it cannot be excluded that another avirulence product (hypothetically Avr4A) is also mutated or lacking in these races.

To prove the model of two or more interacting *R* genes, the expression level of the *Cf-4* gene (Thomas *et al.*, 1997) and the tagged *Cf-4A* gene will be compared in the transposon-tagged plants and their revertants. Complementation studies with the tagged *Cf-4A* gene in MM-Cf0 will elucidate whether the tagged gene is responding specifically to Avr4. The role of the Avr4 elicitor in disease resistance can be tested by an inoculation assay of a modified race 5 of *C. fulvum*, in which the *Avr4* gene has been deleted. If MM-Cf4 plants are still resistant to such a transgenic strain it proves that avirulence products other than Avr4 are involved in the induction of disease resistance conferred by *Cf-4A*.

Experimental procedures

Generation of tagging population

The near isogenic line of the susceptible *L. esculentum* cultivar Moneymaker (MM-Cf0) carrying *Cf-4* (Tichelaar, 1984; I. Boukema, Centre of Genetic Resources (CPRO), the Netherlands) was used (MM-Cf4) to generate the tagging population. The *Ds* transposable element *Ft33* (Rommens *et al.*, 1992), which had been mapped 3 cm from the *Cf-4* locus (Jones *et al.*, 1994), was brought in coupling phase with the *Cf-4* locus. In a separate study (Takken, unpublished results) it has been shown that the *Ft33* transposon exhibits high germinal excision frequency (18%) and a reintegration efficiency of 50% (data obtained from 50 parents analysing 200 progeny plants from each parent).

Progeny plants (394) obtained from a selfing of a heterozygous plant having the *Ds* element in repulsion with the *Cf-4* locus were screened for recombinants having the *Ds* element (PCR using primers Ac4: GGAGCCCAAGCTTTAGCTAGCCC and HPT2: GAATT-CCCAATGTCAAGCACTTCCG) in coupling with the *Cf-4* locus (*C. fulvum* inoculation assay). Only two recombinants were obtained, confirming the tight linkage of *Ds* to the *Cf-4* locus (1 cm). These two (*Cf-4/Ds*) plants were subsequently crossed to 10 plants homozygous for both the *Cf-4* locus and an unlinked *sAc* element itself unable to transpose (Thomas *et al.*, 1994). From the 150 progeny analysed, 89 plants were heterozygous for both *Ds* and *sAc* and homozygous for the *Cf-4* locus. These 89 plants were used as females in a cross to MM-Cf0. The progeny of 20 000 seedlings was screened for susceptible mutants using an inoculation assay with race 5 of *C. fulvum* (IPO-CPRO, the Netherlands).

The transgenic MM-Cf0 plants expressing the *Avr4* gene under control of the 35S cauliflower mosaic virus promoter were a gift from J. Jones (Sainsbury Labs, Norwich, UK).

Race 5 *C. fulvum* inoculations

The infection assay was done as described by De Wit (1977) using a suspension of 10^5 conidia ml⁻¹ obtained from a race 5 *C. fulvum* grown on solid potato dextrose agar. Seedlings were inoculated twice under high humidity (100%), 11 and 20 days after sowing. Plants were scored for susceptibility 2–4 weeks after the first round of inoculation. Susceptibility was scored for by abscission of the

cotyledons and sporulation of the fungus from the lower side of the primary leaves.

Analysis the *avr4* gene of *C. fulvum*

DNA of two different race 5 strains of *C. fulvum* (obtained from C. Thomas, Sainsbury Lab, Norwich, UK, and IPO-DLO, the Netherlands) was isolated according to Van Kan *et al.* (1991). The ORF was sequenced using the primers described by Joosten *et al.* (1994). Sequencing of the PCR products (dye dideoxy automatic sequencing; Applied Biosystems) revealed that the sequence is identical to the sequence determined by Joosten *et al.* (1994).

Apoplastic fluid (AF) obtained from MM-Cf0 plants infected with *C. fulvum* race 5 was isolated as described by De Wit and Spikman (1982). Approximately 30 µl of AF was injected via the lower side of the leaves using a Hamilton syringe. Symptoms were visible 1–3 days after injection, proving the production of the Avr4 elicitor by the fungus. The immunoblots, prepared to detect the ECP2 protein, were made as described by Wubben *et al.* (1994).

PVX::Avr4 infection assay

The *Avr4* gene was expressed in plants using the PVX-based expression system (Chapman *et al.*, 1992). The PVX::Avr4 construct was provided by and used as described by Joosten *et al.* (1997).

RFLP analysis deletion mutants

The molecular RFLP markers TG301, TG51, CT233, CP46 and TG236 (Tanksley *et al.*, 1992) were converted to CAPS (cleavage amplified polymorphic sequence) markers (Bonnema *et al.*, 1996). Specific primers were designed for the sequences present on both ends of the probes; restriction analysis of the PCR products revealed polymorphisms between the *L. esculentum* DNA and the *L. hirsutum* introgression. The positions of these markers were determined in an interspecific *L. esculentum* × *L. pennellii* cross (Tanksley *et al.*, 1992) and at higher resolution in an interspecific *L. esculentum* × *L. peruvianum* cross (Bonnema *et al.*, 1997). For details on the tested markers see Table 2.

DNA isolation and cloning

Tomato DNA was extracted by a method described previously (Van Der Beek *et al.*, 1992). Southern hybridizations and radioactive labelling of probes were done according to standard procedures (Church and Gilbert, 1984; Sambrook *et al.*, 1989). All blots were washed at high stringency $0.1 \times \text{SSC}/0.1\% \text{ SDS w/v}$ at 60°C.

The flanking sequences of the transposed *Ds* element from plant 4 were isolated using plasmid rescue (Rommens *et al.*, 1992). DNA isolated from plant 4 was digested using *Bgl*II, which does not cut in the *Ds* element. The digestion products were circularized with T4 ligase and subsequently electrotransformed into *Escherichia coli* NM554. Sequencing the 1.8 kb insert yielded the 5'-sequence of this tagged gene.

The flanking sequences of the *Ds* element from plant 65 were isolated using anchored PCR (Stuurman *et al.*, 1996) with the following modifications. Plant DNA was digested with *Bgl*II or *Bcl*II instead of *Bst*Y1 and ligation was done in the absence of the restriction enzyme (this was removed by a phenol/chloroform extraction) because the ligated products were recleavable. Authenticity of the obtained products was verified by Southern and

sequence analysis. The obtained products were subcloned in the pGemT-easy vector (Promega USA).

The *Cf-4* specific probe used for Southern blot hybridizations was a 510 bp PCR product obtained from plant 4 using a primer specific for the *Ds* border (LDs10: TTTTACCGACCGTTACCGAC) and a *Cf-4* specific primer (Cf4r1: TTCTTCAAGTTGGGTCCAGC-TTC).

Reverse transcriptase PCR was done to isolate the 3'-end of the *Cf-4* gene. RNA was isolated as described by Van Tunen *et al.* (1988). cDNA synthesis (Gibco BRL) was done using a T₁₇RACE (T₁₇RACE: GACTCGAGTCGACATCGAT₁₇) primer that anneals to the poly(A) tail of the mRNA and adds an extra sequence to the 3'-end. Combining a *Cf-4*-specific primer and a RACE primer (identical to T₁₇RACE without the 17 Ts), *Cf-4* cDNA was specifically amplified.

To identify the absence of introns in *Cf-4*, total RNA, *DNase* treated to remove genomic DNA, was converted to single-strand cDNA using hexamer primers (Gibco BRL). The cDNA obtained was amplified with primers specific for *Cf-4*. Sequencing the obtained PCR products confirmed the identity of the PCR product and the absence of introns. Furthermore, by screening a genomic MM-Cf4 λ Gem11 library, clones were identified containing the *Cf-4A* gene. One of these clones was sequenced to confirm the sequences obtained from plasmid rescue and PCR. Sequence analysis using the BlastN (Altschul *et al.*, 1990) algorithm showed high homology with *Cf-9* (score 3875, probability of 0.0) and the two *Cf-2* genes [924, P(n) 7.1×10^{-114} (*Cf-2-2*) and 9.8×10^{-114} (*Cf-2-1*), respectively].

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EMBL accession number Y12640 (*L. esculentum Cf-4A*).

Note added in proof

The described *Cf-4A* gene was recently also placed in the sequence databases and was referred to in the literature by Parniske *et al.* (1997) and Thomas *et al.* (1997) as the Hcr9-4E gene.