

# The Tomato *Cf-5* Disease Resistance Gene and Six Homologs Show Pronounced Allelic Variation in Leucine-Rich Repeat Copy Number

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The tomato *Cf-2* and *Cf-5* genes confer resistance to *Cladosporium fulvum* and map to a complex locus on chromosome 6. The *Cf-5* gene has been isolated and is predicted to encode a largely extracytoplasmic protein containing 32 leucine-rich repeats (LRRs), resembling the previously isolated *Cf-2* gene, which has 38 LRRs. Three haplotypes of this locus from *Lycopersicon esculentum*, *L. pimpinellifolium*, and *L. esculentum* var *cerasiforme* were compared, and five additional homologs of *Cf-5* were sequenced. All share extensive sequence identity, particularly within the C-terminal portions of the predicted proteins. In striking contrast to the *Cf-9* gene family, six of seven homologs in the *Cf-2/Cf-5* gene family vary in LRR copy number, ranging from 25 to 38 LRRs. *Cf-5* and one adjacent homolog differ by only two LRRs. Recombination events that vary the LRR copy number in this region could provide a mechanism for the generation of new specificities for recognition of different ligands. A recombination breakpoint between the *Cf-2* and *Cf-5* loci was fully characterized and shown to be intragenic.

## INTRODUCTION

Plants have evolved many mechanisms to protect themselves from pathogens. Some of these systems are passive, such as the production of preformed antimicrobial compounds (Osborn, 1996), whereas others involve the induction of defense mechanisms when a specific pathogen is recognized (Hammond-Kosack and Jones, 1996). Dominant or semidominant resistance (*R*) genes in plants are thought to act as molecular receptors for pathogen-derived factors, which are the products of avirulence (*Avr*) genes (reviewed in Staskawicz et al., 1995). This specific interaction was first described for flax and flax rust and led to Flor's classic gene-for-gene hypothesis (Flor, 1946).

The first cloned *R* gene involved in gene-for-gene recognition was *Pto*, which confers resistance in tomato to bacterial spot caused by *Pseudomonas syringae* strains that carry the *AvrPto* gene (Martin et al., 1993). *Pto* encodes a protein kinase that has been shown to interact directly with the *Avr* gene product *AvrPto* (Scofield et al., 1996; Tang et al.,

1996). Several *R* genes have now been isolated, and these form separate classes based on their sequence identity (Bent, 1996; Hammond-Kosack and Jones, 1997). The most abundant class encodes cytoplasmically localized proteins containing a predicted nucleotide binding site and multiple leucine-rich repeats (LRRs) near the C terminus. This class is typified by the *R* genes *N* from tobacco (Whitham et al., 1994), *L6* from flax (Lawrence et al., 1995), and *RPS2*, *RPM1*, and *RPP5* from Arabidopsis (Bent et al., 1994; Mindrinos et al., 1994; Grant et al., 1995; Parker et al., 1997). Another distinct group of *R* genes comprises the tomato *Cf-2*, *Cf-4*, and *Cf-9* genes, which confer race-specific resistance to the leaf mold pathogen *Cladosporium fulvum* (Jones et al., 1994; Dixon et al., 1996; Thomas et al., 1997). The *Cf* genes encode membrane-anchored proteins largely composed of extracytoplasmic LRRs. The *Xa21* gene of rice contains components similar to both the *Pto* and *Cf* genes, encoding a transmembrane receptor-like protein kinase with 23 extracellular LRRs (Song et al., 1995).

Several different *Cf* genes confer resistance to specific races of *C. fulvum* (Stevens and Rick, 1988) and have been bred into cultivated tomato to generate near isogenic lines (see Methods). *Cf-2* and *Cf-9* were identified in *Lycopersicon pimpinellifolium*, and *Cf-5* was identified in the land race *L. esculentum* var *cerasiforme* (Dickinson et al., 1993; Jones et al., 1993). The *Cf-4* gene from *L. hirsutum* and the *Cf-9* gene map to allelic complex loci on the short arm of chromosome 1 (Jones et al., 1994; Thomas et al., 1997).

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A major biological question regarding *R* gene loci concerns whether the mechanisms by which allelic diversity is generated at these loci match pathogen evolution. Detailed comparisons with the allelic locus from susceptible *L. esculentum* have implicated multiple recombination and/or gene conversion events between the *Hcr9* (for homologs of *Cladosporium* resistance gene *Cf-9*) genes in their evolution (Parniske et al., 1997). *Cf-2* and *Cf-5* map to a complex locus on chromosome 6 (Dickinson et al., 1993), and in the course of cloning *Cf-2*, a family of *Hcr2* genes was isolated from both *L. esculentum* and *L. pimpinellifolium* (Dixon et al., 1995, 1996). Here, we report the isolation of the *Cf-5* gene and characterization of the complex locus from three genotypes. By comparing gene sequences, we have identified a region likely to be involved in ligand recognition. This result suggests how unequal recombination could generate novel variation within a specific functional domain of a class of genes. In marked contrast to the *Hcr9* gene family (Parniske et al., 1997), the members of the *Hcr2* gene family differ dramatically in that each has a different number and/or combination of LRRs.

## RESULTS

### Identification of Binary Cosmid Clones Carrying *Cf-5*

A line containing *Cf-2* (*Cf2*) was crossed to a line containing *Cf-5* (*Cf5*) to generate a transheterozygous  $F_1$  plant carrying a single copy of each resistance locus. This  $F_1$  plant was crossed to a line lacking any detectable *Cf* genes (*Cf0*), and ~12,000 testcross progeny were screened for susceptible plants by inoculation with *C. fulvum*. All plants should carry either the *Cf-2* or *Cf-5* resistance genes and therefore be resistant, except when recombination has occurred between the two loci. This strategy led to the isolation of a single susceptible individual (V454) that was shown to carry a recombinant chromosome 6 by using restriction fragment length polymorphism markers closely linked to *Cf-2* (Dixon et al., 1996). When we used a DNA probe hybridizing with part of *Cf-2* as well as with an adjacent, related gene, DNA gel blot analysis revealed a pattern that had some but not all features of the pattern for both *Cf-2* and *Cf-5* genotypes. These data taken together suggested that the *Cf-5* gene is not just closely linked to *Cf-2* but is likely to be part of an allelic locus.

To clone the *Cf-5* gene, we used genomic DNA from plants homozygous for *Cf-4* and *Cf-5* to construct a binary vector cosmid library (see Methods). A 3.8-kb *Xho*I fragment of *Cf-2* was used to screen slot blots and gel blots of cosmid DNA from pools of the library. Several pools were identified and found to contain hybridizing cosmid clones, and individual positive clones were isolated by standard procedures. By a combination of restriction mapping, fingerprinting, and DNA hybridization, the cloned insert DNA in each cosmid was mapped and assembled into a single con-

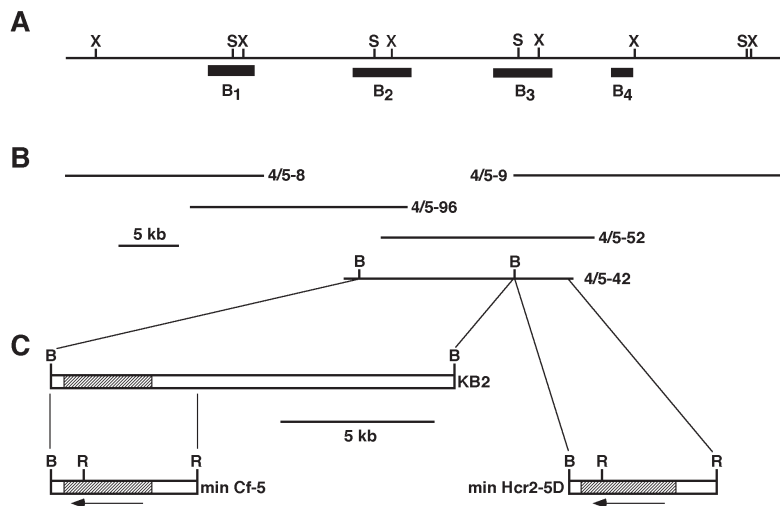
tig (Figure 1A). The assembled contig contained three main *Cf-2*-hybridizing regions ( $B_1$  to  $B_3$ ) and one small region of hybridization ( $B_4$ ). All major *Cf-2*-hybridizing bands on genomic DNA gel blots of *Cf5* plants were represented within this *Cf-5* cosmid contig.

### Complementation in Transgenic Plants Reveals Copy $B_2$ to Be *Cf-5*

Five independent cosmid clones (4/5-8, 4/5-9, 4/5-42, 4/5-52, and 4/5-96; Figure 1B) were mobilized into *Agrobacterium*; however, in each case except for cosmid 4/5-8, the cosmid insert DNA underwent recombination such that some DNA sequence was lost. Each of the cosmids (4/5-9, 4/5-42, 4/5-52, and 4/5-96) that underwent this recombination contained more than one region hybridizing with the *Cf-2* DNA probe. Molecular analysis suggested that recombination between these regions of similarity was responsible for loss of sequence. Mobilization into *Agrobacterium* of a cosmid carrying, for example, more than one region of hybridization with *Cf-2* resulted in the generation of a deleted cosmid containing a single chimeric region of homology to *Cf-2*. To overcome the generation of chimeric genes, we subcloned cosmid 4/5-42 to generate two new cosmids (KB2 and minHcr2-5D; Figure 1C), each containing a single complete region that hybridized with *Cf-2*.

Cosmids 4/5-8, KB2, and minHcr2-5D were mobilized into *Agrobacterium* and transformed into susceptible *Cf0* plants. Transgenic plants were selected and inoculated with a race of *C. fulvum* that contains the  $\beta$ -glucuronidase gene (race 4 GUS). Three of six plants transformed with cosmid KB2 were resistant to *C. fulvum*, whereas none of the seven plants transformed with 4/5-8 and none of the 13 transformed with minHcr2-5D were resistant. One of the resistant KB2-transformed plants was polyploid as a consequence of the transformation process and produced no viable seed. Progeny from the remaining nonpolyploid KB2-transformed plants were screened with races of *C. fulvum* either containing or lacking *Avr5* (race 4 GUS compared with race 5). All progeny were susceptible to *C. fulvum* lacking *Avr5* (race 5), whereas ~75% of progeny from each transformant were resistant to *C. fulvum* carrying *Avr5* (race 4 GUS) (data not shown). These data demonstrate that *Avr5*-dependent resistance to *C. fulvum*, that is, *Cf-5*, must be located within the 13-kb *Bam*HI fragment of cosmid KB2.

To further define the *Cf-5* gene, we subcloned a 4.9-kb *Bam*HI-to-*Eco*RI fragment containing the entire  $B_2$  region of homology to *Cf-2* into the corresponding sites of pCLD04541 to create the plasmid minCf-5 (Figure 1C). Plasmid minCf-5 was mobilized into *Agrobacterium* and transformed into susceptible (*Cf0*) plants. Transgenic plants were selected and inoculated with *C. fulvum* race 4 GUS. Of nine transformants, four were fully resistant, whereas two were partially resistant. This result is entirely consistent with the semidominant nature of *Cf* genes and the implication that expression



**Figure 1.** Physical Map of the *Cf-5* Locus.

(A) Map of *Sacl* (S) and *XhoI* (X) restriction endonuclease sites in the *Cf-5* locus. This map was compiled using data obtained from individual cosmids shown in (B). Filled boxes B<sub>1</sub> to B<sub>4</sub> indicate the specific regions hybridizing with *Cf-2* and the restriction fragment length polymorphism marker MG112.

(B) The cosmids contig spanning the *Cf-5* locus. Horizontal lines represent inserts contained within five independent cosmids clones that hybridize with the *Cf-2* gene probe. Cosmid names are indicated at the ends of each horizontal line. Bar = 5 kb for (A) and (B).

(C) Subcloned regions of cosmids 4/5-42 that were used for direct complementation experiments are represented as rectangular boxes. Hatched regions signify major open reading frames, with the direction of transcription indicated by arrows. Subclone names are indicated at the ends of each horizontal box. Vertical and angled lines indicate the origins of each subcloned region. B, *Bam*HI; R, *Eco*RI. Bar = 5 kb.

levels affect the degree of resistance (Hammond-Kosack and Jones, 1994).

### Structure of the *Cf-5* Gene

The DNA sequence of the *Cf-5* gene carried in plasmid minCf-5 was determined and contained a single major open reading frame encoding a polypeptide of 968 amino acids (Figure 2). Analysis using BESTFIT (Genetics Computer Group, Madison, WI) showed that Cf-5 is 90% identical and 93.3% similar to Cf-2. Like Cf-2, Cf-5 can be divided into seven domains. Domains B, C, and D show homology to the polygalacturonase inhibitor proteins and form the major portion of the predicted protein. Domain C consists entirely of 32 LRRs; several of these can be classified into two subgroups (types A and B). Subgroup A and B LRRs alternate in a pattern similar to that previously described for Cf-2 (Dixon et al., 1996). Compared with Cf-2, Cf-5 lacks a number of LRRs (Figure 2, dashes) and contains a number of other amino acid differences, which are shown in red (Figure 2). As in Cf-4 (Thomas et al., 1997) and the other Hcr9 proteins (Parniske et al., 1997), amino acid differences are most frequent at positions flanking the leucine residues in the LxxLx-LxxN canonical LRR motif (where x can be any amino acid). This region is predicted to form a discriminatory parallel  $\beta$  sheet. Domain A is a predicted signal peptide of 26 amino

acids. Domain F is a potential membrane-spanning region containing 24 uncharged amino acids. Domains E and G are rich in acidic and basic amino acids, respectively, which is consistent with a role in anchoring and orienting the protein within the cell membrane. Domains B to E contain 25 potential N-linked glycosylation sites.

*Cf-5* is likely to be transcriptionally processed in a manner similar to *Cf-2*, because several well-characterized transcriptional control sequences are conserved. A putative TATA box, preceded by two CAAAT boxes, is found only 46 bases upstream of the first initiation codon. The next closest TATA box is >400 nucleotides upstream of the first and lacks any apparent CAAAT boxes. Forty-one bases downstream of the termination codon, there is conservation of sequences corresponding to an intron in *Cf-2*, with the intron-exon boundaries being completely conserved. Similarly, the polyadenylation signal 371 bases downstream of the termination codon is absolutely conserved.

### Comparison of the Cf0, Cf2, and Cf5 Haplotypes

Previously, we reported the isolation and partial characterization of allelic sequences from Cf0 and Cf2 plants (Dixon et al., 1995, 1996). Extensive analysis of these cloned loci, using subcloned regions of the *Cf-2* contig as hybridization probes, has shown that the cloned *Cf-5* contig is colinear

**A** **MMVTSKVFSSLQFFTVFYLFYVAF**

**B** **STEEATALLTWKATFKFNQNSFLASWTTSSNACKDWY**

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	G	V	V	C	L	N	G	R	V	N	T	L	N	I	T	N	A	S	V	I	G	T	L	Y	A	1												
	F	P	F	S	S	L	P	F	L	E	N	L	D	L	S	N	N	N	I	S	G	T	I	P	2													
	P	E	I	G	N	L	T	N	L	V	Y	L	D	L	M	T	N	Q	I	S	G	T	I	P	3													
	P	Q	I	G	S	L	A	K	L	Q	I	I	R	I	F	N	N	H	L	N	G	F	I	P	4													
	E	E	I	G	Y	L	R	S	L	T	K	L	S	L	G	I	N	F	L	S	G	S	I	P	A													
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	E	E	I	G	Y	L	R	S	L	T	K	L	S	L	G	I	N	F	L	S	G	S	I	P	A													
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	A	S	L	G	N	L	N	N	L	F	M	L	Y	L	Y	N	N	Q	L	S	G	S	I	P	B													
	E	E	I	G	Y	L	S	S	L	T	E	L	Y	L	G	N	N	N	S	L	N	G	S	I	P	A												
	A	S	L	G	N	L	N	N	L	F	M	L	Y	L	Y	N	N	Q	L	S	G	S	I	P	B													
	E	E	I	G	Y	L	S	S	L	T	E	L	F	L	G	N	N	N	S	L	N	G	S	I	P	A												
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	S	F	V	C	N	L	T	S	L	E	V	L	Y	M	S	R	N	N	L	K	G	K	V	P	20													
	Q	C	L	G	N	I	S	D	L	H	I	L	S	M	S	N	S	F	R	G	E	L	P	21														
	S	S	I	S	N	L	T	S	L	K	I	L	D	F	G	R	N	N	L	E	G	A	I	P	22													
	Q	F	F	G	N	I	S	S	L	Q	V	F	D	M	Q	N	N	K	L	S	G	T	L	P	23													
	T	N	F	S	I	G	C	S	L	I	S	L	N	L	H	G	N	E	L	A	D	E	I	P	24													
	R	S	L	D	N	C	K	K	L	Q	V	L	D	L	G	N	N	L	N	D	T	F	F	25														
	M	M	L	G	T	L	P	E	L	R	V	L	R	L	T	S	N	K	L	H	G	P	I	R	S	26												
	G	A	E	I	M	F	P	D	L	R	I	I	D	L	S	R	N	A	F	S	Q	D	L	P	27													
	S	L	F	E	H	L	K	G	M	R	T	V	D	K	T	M	E	E	P	S	Y	E	S	Y	D	D	S	V	V	V	V	T	K	G	L	E	L	28
	E	I	V	R	I	L	S	L	Y	T	I	I	D	L	S	S	N	K	F	E	G	H	I	P	29													
	S	V	L	G	D	L	I	A	I	R	V	L	N	V	S	H	N	A	L	Q	G	Y	I	P	30													
	S	S	L	G	S	L	S	I	L	E	S	L	D	L	S	F	N	Q	L	S	G	E	I	P	31													
	Q	Q	L	A	S	L	T	F	L	E	V	L	N	S	H	N	T	L	Q	G	C	I	P	32														

**D** **QGPQFRTEFESNSYEGNDGLRGYPVSKGCGK**

**E** **DPVSEKNYTVSALEDQESNSEFFNDFWK**

**F** **AALMGYSGGLCIGISIIYILISTG**

**G** **NLRWLARIIEELEHKIIIVQRKKQGRGQRNYRRNRNF**

**Figure 2.** Primary Structure of the Cf-5 Protein.

The amino acid sequence predicted from the sequence of the Cf-5 gene is shown divided into seven domains (A to G), as described in the text. The sequence has been aligned with Cf-2; amino acid differences are shown in red, and dashes indicate the absence of amino acids in Cf-5. In domain C, the conserved L residues of the LRRs are often replaced by V, F, I, or M. The positions of these conserved residues are marked by asterisks at the top of domain C. Numbers to the right of domain C indicate the specific LRR number, and letters A and B to the right of the sequence indicate the type of LRR (see text). Underlined residues indicate potential N-linked glycosylation sites. Vertical lines bracket the region predicted to form the solvent-exposed  $\beta$  sheet. The Cf-5 gene sequence has GenBank accession number AF053993.

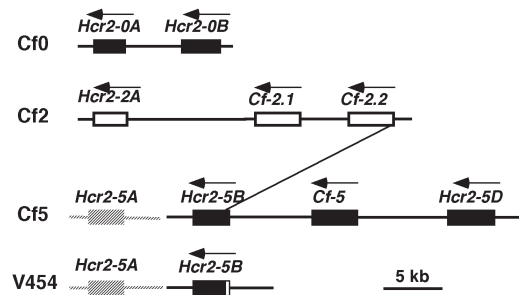
with these other loci (Figure 3). Figure 3 shows boxed regions representing sequences within these three loci that hybridize with the Cf-2 gene probe and that have been designated Hcr2 genes. The second digit denotes the genotype, and the final letter designation refers to the relative position within each haplotype. When this nomenclature is used, Cf-5 can also be referred to as Hcr2-5C.

DNA gel blot analysis revealed that all but one of the Hcr2 genes at the Cf-5 locus had been isolated. Hcr2-5A, corresponding to homologs Hcr2-0A and Hcr2-2A, was not cloned. A DNA probe upstream of Hcr2-2A hybridizes with the adjacent region of Hcr2-0A but not to regions adjacent to the other Hcr genes or Cf genes in the three contigs. This same probe detected sequences on DNA gel blots of genomic DNA from plants carrying Cf-5, indicating the presence of an Hcr2-5A (data not shown). It is likely that Hcr2-0A, Hcr2-2A, and Hcr2-5A are truly orthologs within the Cf gene family.

**Sequence of Other Hcr Genes**

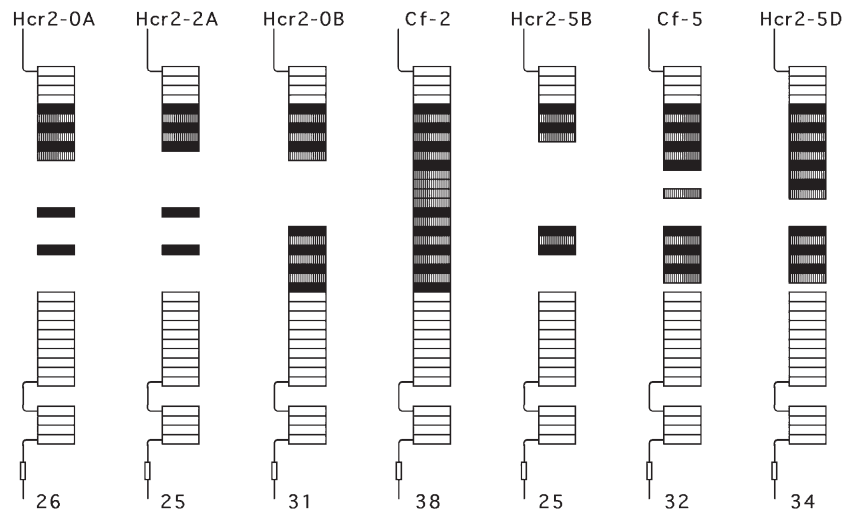
The individual Hcr2 genes from each cloned contig were subcloned and sequenced. For each, a single major open reading frame, with homology to the Cf-2 and Cf-5 genes, was maintained (Figure 4). Only the small region of homology designated B<sub>4</sub> in the Cf-5 contig (Figure 1) carried an incomplete homolog. B<sub>4</sub> contained sequences corresponding only to the most N-terminal regions and lacks any homology to the remainder of the Cf genes (data not shown).

All of the Hcr2s sequenced (Hcr2-0A, Hcr2-0B, Hcr2-2A, Hcr2-5B, and Hcr2-5D) encode polypeptides that can be divided into the same seven general domains as Cf-2 and Cf-5 (A to G). The most notable differences between all of these predicted proteins are that they each contain different numbers and/or combinations of LRRs and that this variation occurs within the region of highly conserved alternating A/B-type



**Figure 3.** Analysis of Allelic Regions of Chromosome 6.

Shown is a schematic representation of allelic regions of chromosome 6 from the near-isogenic lines Cf0, Cf2, and Cf5 and the rare recombinant between Cf-2 and Cf-5 (V454). Boxed regions represent the open reading frames of the Cf genes and their homologs; arrows indicate the direction of transcription. Filled boxes show DNA derived from *L. esculentum*, open boxes show DNA derived from *L. pimpinellifolium*, and hatched regions indicate DNA identified by DNA gel blot analysis but not cloned. The diagonal line between Cf-2.2 and Hcr2-5B shows the approximate position of the recombination event between these two genes. The bar indicates 5 kb; however, distances for the uncloned region (broken lines) are not known and cannot be compared.



**Figure 4.** Schematic Representation of Proteins Encoded by *Cf-2*, *Cf-5*, and Five *Hcr2* Genes.

For each encoded protein, vertical blocks indicate predicted transmembrane regions and single horizontal bars indicate LRRs. Solid bars signify type A repeats, and vertically striped bars signify type B repeats. Open bars indicate LRRs that have sequences that do not match the type A or B consensus. LRRs for each protein are aligned to the largest family member (*Cf-2*). Numbers below each protein indicate the number of LRRs predicted for each. The GenBank accession numbers for the *Hcr* gene sequences are AF053994 for *Hcr2-0A*, AF053995 for *Hcr2-0B*, AF053996 for *Hcr2-2A*, AF053997 for *Hcr2-5B*, and AF053998 for *Hcr2-5D*.

LRRs (Figure 4). Apart from the dramatic variation in the number of LRRs, several other amino acid differences exist between the *Cf-2*, *Cf-5*, and *Hcr* genes, although these tend to be distributed mainly throughout domain C. Significantly, *Hcr2-5D* is almost identical to *Cf-5*, only differing by a single amino acid substitution adjacent to two additional LRRs (Figure 5).

Transgenic plants containing *Hcr2-5D* do not confer resistance to *C. fulvum*, despite the near identity to *Cf-5*. To confirm this result, expression of *Hcr2-5D* was examined. The high degree of identity between *Hcr2-5D* and the *Hcr2-0B* homolog in the susceptible Cf0 line used for transformation precluded the use of RNA gel blot analysis for this purpose. Instead, reverse transcriptase-polymerase chain reaction (RT-PCR) experiments were performed with poly(A)<sup>+</sup> RNA extracted from cotyledons of progeny from two *Cf-5* and three *Hcr2-5D* transformants as well as from the original nontransgenic Cf5 line (Figures 6A and 6B). Primers CF5F2 and CF5R2, designed to anneal to a conserved 3' portion of *Cf-5* and *Hcr2-5D* but not to *Hcr2-0B*, amplified a product of ~1.6 kb from Cf5 genomic DNA but not from Cf0 genomic DNA, thus showing their specificity. These primers amplified a smaller product of ~1.4 kb from all RNA samples, indicating expression of the transgenes. The size difference between the products from genomic and cDNA confirms the presence of the predicted intron in the 3' untranslated sequences and proves that the amplification is specific and not the result of contaminating genomic DNA (Figure 6A). Primers 2-5CF and 2-5CR were designed for sequences flanking the two additional LRRs in *Hcr2-5D* and therefore reveal characteristic size poly-

morphisms for the two genes and transcripts corresponding to *Cf-5* and *Hcr2-5D*. These primers clearly reveal the expression of both transcripts in the original nontransgenic line Cf5 and the expression of individual transcripts in each of the five transgenic plants examined (Figure 6B).

#### Characterization of the Recombination Event in V454

Detailed DNA gel blot analysis of the recombinant V454 suggested that the crossover event between the *Cf-2* and *Cf-5* parental chromosomes was unequal and resulted in the loss

Cf-5	Hcr2-5D
ASLGNLNNLSFLYLYNNQLSGSIP	ASLGNLNNLSFLYLYNNQLSGSIP
EEIGYLRSLTKLSLGINFLSGSIP	EEIGYLRSLTKLSLGINFLSGSIP
ASLGNLNNLSRLDLYNNKLSGSIP	ASLGNLNNLSRLDLYNNKLSGSIP
EEIGYLRSLTYLDLGENALNGSIP	EEIGYLRSLTYLDLGENALNGSIP
SSLGNLNNLSRLDLYNNKLSGSIP	<b>ASLGNLNNLFMLYLYNNQLSGSIP</b>
EEIGYLRSLTYLDLGENALNGSIP	<b>EEIGYLRSLTYLDLGENALNGSIP</b>
ASLGNLNNLFMLYLYNNQLSGSIP	<b>ASLGNLNNLSRLDLYNNKLSGSIP</b>
EEIGYLRSLTYLDLGENALNGSIP	<b>EEIGYLRSLTYLDLGENALNGSIP</b>
ASLGNLNNLFMLYLYNNQLSGSIP	<b>ASLGNLNNLFMLYLYNNQLSGSIP</b>
EEIGYLRSLTYLDLGENALNGSIP	<b>EEIGYLRSLTYLDLGENALNGSIP</b>

**Figure 5.** Amino Acid Sequence for Analogous Regions of *Cf-5* and *Hcr2-5D*.

Shown is an alignment of portions of the predicted *Cf-5* and *Hcr2-5D* proteins from LRR 8 to LRRs 15 and 17, respectively. Amino acid differences and changes between the two are highlighted on a black background.

of several *Hcr2* genes. The V454 recombinant contains *Hcr2-5A* and a chimeric gene consisting mainly of *Hcr2-5B* and a small part of *Cf-2.2* (Figure 3). Using the sequence of these two genes, it was possible to design specific synthetic oligonucleotide primers for use in PCR that could amplify the site of recombination in V454. Primer V3F was designed to anneal to *Hcr2-5B* and no other sequenced homolog in Cf2 or Cf5 plants, whereas primer V3R was designed to fulfill the same specifications and anneal only to the *Cf-2* genes (see Methods). Used in combination, these primers amplified a novel product in the recombinant plant V454 that did not amplify from Cf2 or Cf5 plant DNA. Sequencing of the novel product indeed confirmed that V454 carried a chimeric sequence of *Hcr2-5B* and *Cf-2.2* (Figure 7). The point of recombination can be located only to a region between the two closest flanking heterologous nucleotides, which in this case span a region of 107 identical nucleotides. This recombination event occurred within sequences encoding parts of domains A and B (Figure 7). In contrast, previously described recombination events between the *Cf-9* and *Cf-4* loci were in intergenic regions (Parniske et al., 1997).

## DISCUSSION

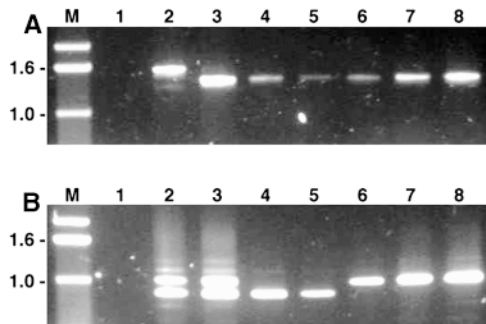
### Structure of the *Cf-5* Gene Product

The *Cf-5* gene closely resembles the *Cf-2* genes because it encodes a membrane-anchored extracytoplasmic protein,

as might be predicted for a receptor evolved to detect an extracellular pathogen. Like Cf-2, an extensive region of Cf-5 comprises extracytoplasmic LRRs (Dixon et al., 1996; Jones and Jones, 1996), 28 of which are exactly 24 amino acids in length. In addition, a region within domain C contains highly conserved LRRs, which can be classified into subgroups (A and B) based on their sequence. Type A repeats have the consensus EEIGL(R/S)SLTXLXLGXNXL(N/S)GSIP, whereas type B repeats have the consensus ASLGNLNNL(S/F)XLXLYNN(Q/K)LSGSIP. Seven of each of these repeats form an alternating region within domain C. The uninterrupted region of LRRs is proposed to give the Cf proteins a highly ordered reiterated structure, although to date, the crystal structure of an extracellular-type LRR has not been determined. The Cf LRRs are predicted to form a stacked structure with the Lxx-LxLxx part of each LRR contributing to a solvent-exposed face composed of parallel  $\beta$  sheets (Jones and Jones, 1996). Indeed, modeling studies with a Cf-2 LRR strongly support this hypothesis (Kajava, 1998). Interestingly, within the LxxLxLxx region of the types A and B LRRs, 43% of the amino acids vary between Cf-5 and Cf-2, whereas outside this region but still within the A/B repeat region, only 9% differ. The leucine residues never vary, but the amino acids that are predicted to be solvent exposed in this region of parallel  $\beta$  sheet are hypervariable (Figure 2). Detailed analysis of Cf-9 and many Hcr9 proteins indicate that the corresponding region is hypervariable and potentially contributes to recognition specificity (Parniske et al., 1997; Thomas et al., 1997).

### Structure of the *Cf-0*, *Cf-2*, and *Cf-5* Allelic Loci

The allelic loci from the three genotypes Cf0, Cf2, and Cf5 each carry a different number of tandemly arranged *Cf*-like genes (two, three, and four, respectively). It is likely that these genes evolved from a common progenitor that existed before speciation and that duplication and unequal exchange events have resulted in the present gene arrangements. Phylogenetic tree analysis of Cf-2, Cf-5, and the Hcr proteins clearly shows that the predicted proteins fall into two classes: Hcr2-0A and Hcr2-2A form one group, and the Cf and remaining Hcr proteins form the second (Figure 8). Figure 4 demonstrates the relatedness of Hcr2-0A and Hcr2-2A and shows that they share the most similar combination of LRRs. BESTFIT analysis of the nucleotide sequences further clarifies this subclassification. Sequences 5' of the translation initiation codon in *Hcr2-0A* and *Hcr2-2A* share >91% identity, whereas no significant homology can be found between these sequences and the upstream regions from any of the other *Cf* or *Hcr* genes. *Hcr2-0A* and *Hcr2-2A* originate from different *Lycopersicon* species, yet the similarity they share is greater than with the adjacent related genes in their respective species, suggesting that duplication and divergence must have occurred before speciation.



**Figure 6.** Analysis of Transgene Expression by RT-PCR.

PCR was performed with either genomic DNA (Cf0, lanes 1; Cf5, lanes 2) or cDNA derived from cotyledons from progeny of various plants (Cf5, lanes 3; two independent *Cf-5* transformants, lanes 4 and 5; three independent *Hcr2-5D* transformants, lanes 6 to 8). Lengths of DNA markers (M) are indicated at the left in kilobases.

**(A)** PCR amplification of conserved 3' sequences of *Cf-5* and *Hcr2-5D*, using primers CF5F2 and CF5R2.

**(B)** PCR amplification of sequences from *Cf-5* and *Hcr2-5D* encompassing the difference of two LRRs, using primers 2-5CF and 2-5CR.



domain. Exactly the same phenomenon of hypervariability is clearly apparent when Cf-5 is compared with Cf-2 (Figure 2).

In striking contrast to the Hcr9 proteins, the Hcr2 proteins all contain different numbers and/or combinations of types A and B LRRs (Figure 4). This is probably a consequence of the extremely regular sequence of the LRR motifs within the *Hcr2* genes that provides increased scope for unequal exchange events or slippage during DNA replication. The LRRs of the Hcr9 proteins are much less regular in length and sequence, reducing the possibility of DNA misalignment before recombination. However, unequal crossover events must still be possible within the *Hcr9* genes because *Cf-4* encodes two fewer LRRs than does *Cf-9* (Thomas et al., 1997).

A general feature of the Hcr2 proteins is that they have alternating types A and B LRRs. However, Hcr2-2A contains three consecutive A-type repeats, and Cf-2 contains four consecutive B-type repeats. This may reflect that either one or more ancestral Hcr2 proteins had stretches of multiple type A or B repeats or that on occasion, unequal recombination can occur when types A and B repeats align, perhaps at the nucleotides encoding the shared amino acids GSIP. It may be possible to examine this by selecting new recombination events within the *Cf-2/Cf-5* locus. Alternatively, the presence of several consecutive type A or B LRRs may support the idea of DNA slippage during replication. The mechanisms of recombination and slippage are not mutually exclusive, and both could contribute to the observed variation in LRR copy number.

It is probable that many recombination events between *Cf-2* and *Cf-5* were not detected during the selection of V454, because the screen was only for completely susceptible individuals. Any recombination events giving rise to resistant plants would have been missed. Recombination in plants can occur between small regions of identity. Analysis of 130 intragenic recombinants of the maize *bronze* gene identified one recombination junction located at a 19-bp interval and six others at intervals of <30 bp (Dooner and Martinez-Férez, 1997).

### Inferences about Function from Hcr2 Protein Structure

The Cf proteins are thought to work as molecular receptors that enable the plant to detect the presence of *C. fulvum* Avr gene products and then initiate the activation of specific defense responses. The C-terminal portions of the Cf proteins show greatest similarity and are proposed to have a role in signaling (Dixon et al., 1996; Thomas et al., 1997). In the absence of an extensive or highly conserved cytoplasmic region, any signaling role would most likely involve interaction with a protein that is integral to or closely associated with the plasma membrane. Central to this model for Cf protein function is the concept that the highly variable regions within the LRRs are responsible for recognition of pathogen-encoded avirulence determinants either directly or indirectly through some coreceptor. Thomas et al. (1997) have shown that the Cf-4 and Cf-9 proteins have identical C termini yet recognize distinct avirulence factors Avr4 and Avr9. Here, we

describe Cf-5 and Hcr2-5D, which differ in only a small region of domain C yet have distinct specificities: Cf-5 confers resistance against *C. fulvum* races carrying the *Avr5* gene, whereas Hcr2-5D does not. We propose that the presence of the two additional LRRs and/or the single amino acid change in Hcr2-5D disrupts a region involved in specific recognition of Avr5 (Figure 5), thus defining this region with unprecedented precision. The single amino acid change is immediately adjacent to the two additional LRRs; therefore, together they constitute only a single change at the nucleotide level. Based on the predictions of Parniske et al. (1997), the single residue change adjacent to the two additional LRRs in Hcr2-5D is unlikely to alter any recognition specificity because it falls outside the xxLxLxx domain hypothesized to form the discriminatory surface. In addition, the serine-to-alanine change is a conservative substitution. A more plausible explanation is that specific amino acids within LRRs both above and below the point of the two-LRR insertion in Hcr2-5D are required to provide Avr5 specificity in Cf-5. The insertion of two additional LRRs into the middle of any recognition domain, as in Hcr2-5D, would alter the spacing between amino acids on the discriminatory surface, thus disrupting any specific interaction/recognition event. The presence of very highly conserved types A and B LRRs within domain C increases the likelihood of unequal crossover events in a region of the Cf genes responsible for encoding recognition specificities. This would shuffle LRRs, mixing existing variability to generate new Hcr genes encoding novel combinations of LRRs and potentially new recognition capabilities.

Thomas et al. (1997) and Parniske et al. (1997) propose that the consecutive LRRs within the Cf proteins create a structure upon which the variant amino acids within each repeat confer recognition capacity. Cf-4 and Cf-9 either directly or indirectly recognize fungal avirulence determinants encoded by *Avr4* and *Avr9*, respectively. These encode secreted proteins of 28 and 106 amino acids (van Kan et al., 1991; Joosten et al., 1994), of which *Avr9* is known to form a small cystine-knot protein (Vervoort et al., 1997). If the interaction is direct, then these small peptides might only contact a few of the LRRs in the recognition domains of Cf-4 and Cf-9. Given the large number of consecutive LRRs in the Cf proteins, each Cf protein would probably have the potential to recognize many different ligands, thereby increasing the potential of each Cf gene as an R gene. The *RPM1* gene of Arabidopsis recognizes two distinct pathogen avirulence genes, *avrB* and *avrRPM1* (Bisgrove et al., 1994). Two Hcr genes of Cf-9 have the capacity to specify Avr9-independent resistance against *C. fulvum* in adult plants and therefore must contain additional recognition specificities (Parniske et al., 1997).

### Recombination and the Generation of New Resistance Specificities

Many of the features of the *Hcr2* and *Hcr9* loci are reflected in other characterized R gene loci, most notably local multi-



gene families, internal duplication of LRR-encoding sequences, and intralocus recombination.

Genetically linked multigene families are present within several other *R* gene loci, such as *Pto* (Martin et al., 1993), *N* (Whitham et al., 1994), *Xa21* (Song et al., 1995, 1997), *M* (Anderson et al., 1996), and *RPP5* (Parker et al., 1997). In addition, several *R* gene homologs have been mapped to clusters in Arabidopsis and potato (Leister et al., 1996; Botella et al., 1997). The flax *M* gene is part of a gene family comprising ~15 members, all of which map to a locus of <1 Mb (Anderson et al., 1996). However, not all *R* genes map to multigene loci. The flax *L6* gene, which is a homolog of *M*, maps to a single gene locus that has multiple alleles (Lawrence et al., 1995; Anderson et al., 1996). *RPS2* (Mindrinos et al., 1994) and *RPM1* (Grant et al., 1995) map to single loci, and *RPM1* has no related homolog at the allelic locus in susceptible lines.

Duplication of LRR-encoding domains has been observed within several *R* genes. In *RPP5*, four probable duplication events have been inferred, and an allele of the *RPP5* resistance gene has been identified that contains an intragenic duplication of four LRRs (Parker et al., 1997). In *L6* and *M*, there has been at least one large duplication within the LRR-encoding region. The *L2* allele seems to have undergone a second duplication event so that it now contains four copies of this large-order repeat unit (Ellis et al., 1997). These observations demonstrate the existence of mechanisms that generate duplications and thus their potential, although definitive proof of their specific contribution is still lacking, to create novel resistance specificities.

The *Rp1* locus of maize provides a good example of unequal recombination at a complex locus; lines carrying three *Rp1* genes with different specificities can be generated (reviewed in Hulbert, 1997). Also, there is a correlation between unequal recombination, gene conversion, and the generation of new specificities, although the molecular basis for this has not been examined.

The presence of multiple genes within complex loci permits intergenic as well as intragenic unequal exchange events to generate new alleles. After any initial duplication event, one gene can be maintained to perform its normal role while variation accumulates in the other. As with the *Hcr9* proteins, the solvent-exposed amino acids in the presumed  $\beta$  sheet of the LRR domain are hypervariable. Recombination and gene conversion probably contribute the variation at both *Hcr2* and *Hcr9* loci. However, because the *Hcr2* proteins contain extremely regular repeating LRR motifs, there is increased scope for intragenic unequal exchange events. As a consequence, there exists dramatic variation in the number and combination of LRRs within the *Hcr2* ligand recognition domain, even though the mechanism of generation of variation (recombination) is the same as for *Hcr9* genes. It will be interesting to determine the extent of these contrasting modes of *Hcr2* and *Hcr9* gene evolution within other complex *R* gene loci.

## METHODS

### Plant Stocks and Inoculation Procedures

The near isogenic lines of *Lycopersicon esculentum* cv Moneymaker carrying either *Cf-2* (*Cf2*) or *Cf-5* (*Cf5*) and the original Moneymaker line carrying no detectable resistance genes to *Cladosporium fulvum* (*Cf0*) were obtained from R. Oliver (University of East Anglia, Norwich, UK). Assays for disease resistance were performed as described by Dickinson et al. (1993).

### Construction of Cosmid Library

Genomic DNA from plants homozygous for *Cf-5* and *Cf-4* was extracted as described by Carroll et al. (1995), except that high molecular weight DNA was recovered by spooling rather than precipitation. All subsequent techniques were performed according to Sambrook et al. (1989), unless otherwise stated. Further purification of genomic DNA was performed on a cesium chloride-ethidium bromide gradient. This DNA was partially digested with *Mbol*, dephosphorylated, and size fractionated twice on 10 to 40% (w/v) sucrose gradients. Fractionated insert DNA (1  $\mu$ g) was ligated at 12°C for 18 hr in a total volume of 10  $\mu$ L with 250 ng of *Bam*HI-digested pCLD04541 binary cosmid vector. Ligated DNA was packaged using commercial extracts (Gigapak, Stratagene) according to the manufacturer's instructions and transfected into SURE tetracycline-sensitive *Escherichia coli* (Stratagene).

### Tomato Transformation

All DNA for transformation into plant cells was cloned into the binary cosmid vector pCLD04541 and mobilized into *Agrobacterium tumefaciens* LBA4404. Transformation of tomato cotyledons (variety Moneymaker *Cf0*) and plant regeneration was performed essentially as described by Fillatti et al. (1987).

### DNA Subcloning

Cosmid KB2 was generated by subcloning a 13-kb *Bam*HI fragment of cosmid 4/5-42 into the *Bam*HI site of the binary cosmid vector pCLD04541. Sequence analysis and comparison with the published *Cf-2* gene showed that the two *Bam*HI sites in cosmid 4/5-42 correspond to a similar site downstream of the polyadenylation site in *Cf-2*. Transformed plants that were resistant to *C. fulvum* had previously been generated using *Cf-2* constructs, which only contained sequences up to this conserved *Bam*HI site (Dixon et al., 1996).

Sequences containing only the *Hcr2-5D* region or only the *Cf-5* region were subcloned into the pCLD04541 vector as *Bam*HI-EcoRI fragments to create the cosmids minHcr2-5D and minCf-5, respectively. Due to the presence of an internal EcoRI site in each region, these subclonings were achieved using partial digestion with EcoRI to obtain the desired fragments.

### DNA Sequencing

All templates for sequencing were prepared from clones containing randomly sheared target DNA generated by nebulization. The ends

of sheared fragments were repaired using T4 DNA polymerase (Pharmacia) and subsequently cloned into dephosphorylated HincII-digested pUC119 or M13mp18 (Sambrook et al., 1989). Standard sequencing reactions were performed using the PRISM Ready Reaction Terminator Cycle Sequencing system (Perkin-Elmer). Double-stranded plasmid DNA for sequencing was isolated according to instructions given in the PRISM sequencing protocol handbook (Perkin-Elmer). Single-stranded DNA was prepared according to standard procedures. The reaction products were run on Applied Biosystems (Foster City, CA) 373 and 377 DNA sequencers, and data were assembled using UNIX versions of the Staden programs package (Roger Staden, MRC, Cambridge, UK), including TED and XBAP. Analysis of assembled sequences was performed using Genetics Computer Group (Madison, WI) programs. Extended sequencing reads of selected template DNA preparations were obtained using a dye primer Thermo sequencing kit (Amersham Pharmacia, Little Chalfont, UK) in conjunction with fluorescently labeled standard forward and reverse sequencing primers (Li-Cor, Lincoln, NE). Reactions were run on a Li-Cor DNA sequencer model 4000L, and data were analyzed as previously described.

#### RNA Extraction and First-Strand cDNA Synthesis

Total RNA was extracted exactly as described by Dixon and Hammond-Kosack (1997). One milligram of oligo(dT)<sub>25</sub> Dynabeads (Dyna, Oslo, Norway) was used according to the manufacturer's instructions to select poly(A)<sup>+</sup> RNA for each sample. Poly(A)<sup>+</sup> RNA was recovered using 10  $\mu$ L of elution buffer, and half of each sample was used for the synthesis of first-strand cDNA using Expand reverse transcriptase (Boehringer Mannheim) according to the manufacturer's instructions. Before polymerase chain reaction (PCR), cDNA was purified from oligo(dT) and other cDNA synthesis components using QIAquick spin columns (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

#### PCR

PCR was performed using 35 cycles of amplification. Primers CF5F2 (5'-GTAATATCAGTGACCTTCACA-3') and CF5R2 (5'-ATTTCCAACTGAAGAAAAG-3') were used at an annealing temperature of 55°C to amplify the 3' conserved regions of *Cf-5* and *Hcr2-5D* containing the intron in the untranslated tails. The region spanning the leucine-rich repeat (LRR) variability between *Cf-5* and *Hcr2-5D* was amplified using the primers 2-5CF (5'-GCTATCTTTGGGTATCAACTT-3') and 2-5CR (5'-AGATGACATCGACAAAATGTG-3') at an annealing temperature of 58°C. To achieve amplification of specific products from the recombinant plant V454, we used the primers V3F (5'-ACATGTGAGAGAAGACATTACG-3') and V3R (5'-ACAAGTTGGTCATATTGCCCAA-3') at an annealing temperature of 49°C.

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**The Tomato *Cf-5* Disease Resistance Gene and Six Homologs Show Pronounced Allelic Variation in Leucine-Rich Repeat Copy Number**

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