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Novel Disease Resistance Specificities Result from Sequence Exchange between Tandemly Repeated Genes at the *Cf-4/9* Locus of Tomato

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Summary

Tomato *Cf* genes confer resistance to C. fulvum, reside in complex loci carrying multiple genes, and encode predicted membrane-bound proteins with extracytoplasmic leucine-rich repeats. At least two *Cf-9* homologs confer novel C. fulvum resistance specificities. Comparison of 11 genes revealed 7 hypervariable amino acid positions in a motif of the leucine-rich repeats predicted to form a β-strand/β-turn in which the hypervariable residues are solvent exposed and potentially contribute to recognition specificity. Higher nonsynonymous than synonymous substitution rates in this region imply selection for sequence diversification. We propose that the level of polymorphism between intergenic regions determines the frequency of sequence exchange between the tandemly repeated genes. This permits sufficient exchange to generate sequence diversity but prevents sequence homogenization.

Introduction

Multicellular eukaryotic organisms activate defense responses when they perceive pathogens. Trypanosomes and bacteria can alter the structure of components that are recognized by the host (e.g., Maskell et al., 1993; Vanhamme and Pays, 1995). The rate at which sequence variability is generated in key genes of the interacting organisms appears to determine the long term outcome of the contest between host and pathogen. In vertebrates this is exemplified by the variability of the antigen recognition site of antibodies and major histocompatibility complex class I and II proteins (Parham and Ohta, 1996; Ramsden et al., 1997). A similar requirement for sequence variability in proteins that determine specific recognition seems to exist in plants, which also possess inducible defense systems to resist microbial attack. In many plant/pathogen interactions, the rapid onset of defense responses relies on a recognition event controlled by an avirulence (*Avr*) gene in the pathogen and by a corresponding disease resistance (*R*) gene in the plant. *R* genes are postulated to encode receptors that, upon binding of the *Avr* gene product, elicit the defense response. Consistent with their proposed receptor function, the products of many *R* genes contain leucine-rich repeats (LRRs) (Jones and Jones, 1997) that can participate in protein–protein interactions (Kobe and Deisenhofer, 1995).

Mutations in fungal *Avr* genes enable the fungus to escape recognition by the corresponding plant *R* genes (Van den Ackerveken et al., 1992; Joosten et al., 1994; Rohe et al., 1995). Therefore, the rapid evolution of novel resistance gene specificities appears to be crucial for the host, and the mechanisms by which this variability is generated are of major interest. The genomic organization of *R* gene loci may be important in determining the rate at which sequence variability evolves. The *M* rust resistance locus in flax (Anderson et al., 1997), the *Rp1* rust resistance complex in maize (Sudupak et al., 1993), and the *Dm3* downy mildew locus in lettuce (Anderson et al., 1996) all consist of multiple genetically linked resistance specificities. High rates of spontaneous meiotic loss of *R* gene specificities at the *Rp1* complex suggest that they reside in an unstable region of the genome. *Rp1* allele instability and the generation of novel Rp1 specificities are often associated with flanking marker exchange, so unequal crossing-over and gene conversion events are likely to be involved (Sudupak et al., 1993; Richter et al., 1995).

The tomato–Cladosporium fulvum pathosystem is particularly amenable to determining the molecular mechanisms involved in the evolution of novel specificities at complex *R* gene clusters. Three *Cf* genes, all conferring different recognition specificities (Jones et al., 1994; Dixon et al., 1996; Thomas et al., 1997), as well as the fungal avirulence genes *Avr4* and *Avr9* have been cloned (Van den Ackerveken et al., 1992; Joosten et al., 1994). The *Cf-2*, *Cf-4*, and *Cf-9* genes of tomato are predicted extracellular membrane anchored proteins. The bulk of the gene products of the cloned *Cf* genes comprise 25–38 extracytoplasmic LRRs (Jones et al., 1994; Dixon et al., 1996). This structure led to the hypothesis that they are the direct receptors of the corresponding *Avr* gene products (Jones et al., 1994).

The *Cf-4* gene originating from Lycopersicon hirsutum and the *Cf-9* gene originating from L. pimpinellifolium map at a complex locus on the short arm of chromosome 1 of tomato (Balint-Kurti et al., 1994). In this report, we show that only a single *Hcr9* gene (*H*omolog of *C*ladosporium *R*esistance gene *Cf-9*) is found at this locus in a disease-susceptible cultivar of L.esculentum (Moneymaker Cf0). Furthermore, we found that other members of the *Cf-9* gene family confer adult plant resistance. We characterized the entire sequence of all three haplotypes gaining insight into the molecular mechanisms by which novel recognitional specificities are generated.

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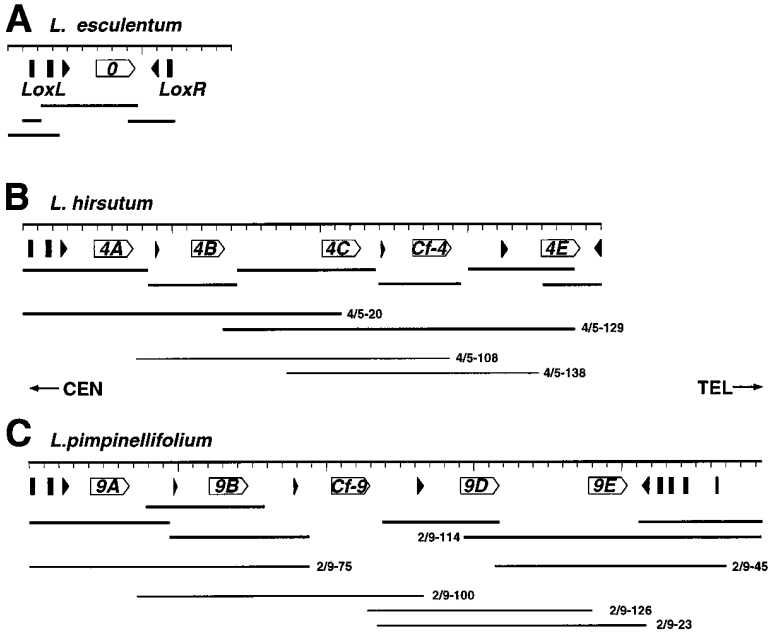


Figure 1. Physical Maps of Three Sequenced Haplotypes at the *Cf-4/9* Locus

The phage clone carrying *Hcr9-0* from the disease-susceptible line Cf0 (A) aligned with cosmid contigs encompasing the *Cf-4* (B) and *Cf-9* genes (C). A 1 kb scale is shown by ticks below physical maps. The position of the intronless *Hcr9* ORFs and the transcriptional orientation are indicated by arrows. The position of *LoxL* and *LoxR* exons are shown by filled boxes and triangles. Sequences homologous to the 3′ exon of *LoxL* interspersed between *Hcr9s* are also shown by filled triangles. Cosmids and subclones used as sequencing templates or to initiate random sequencing are indicated by bold lines. To examine the *R* gene phenotype of *Hcr9* genes (see Figure 6), cosmids 2/9-45 and 2/9-75 were transformed into Cf0. Cen and Tel indicate the orientation of the locus relative to the centromere and telomere of chromosome 1, respectively.

Results

Organization of the *Cf-4* , *Cf-9* , and *Cf-0* Locus in Near Isogenic Lines of Tomato

In the near isogenic L. esculentum lines Cf4, Cf9, and Cf0 (Tigchelaar, 1984), three different haplotypes of the *Cf-4/9* resistance gene locus are represented. The Cf4 and Cf9 lines carry the resistance genes *Cf-4* or *Cf-9* from L. hirsutum and L. pimpinellifolium, respectively, introgressed into Cf0 on the short arm of chromosome 1 (Balint-Kurti et al., 1994). The Cf0 line carries L. esculentum DNA, conferring no known resistance to C. fulvum. The *Cf-4* and *Cf-9* genes are members of a large gene family of which a number are tightly linked to the *Cf-4/9* locus (Jones et al., 1994; Thomas et al., 1997; and data not shown). A series of overlapping cosmid and phage clones were identified using a 700 bp probe from the 5′ region of the *Cf-9* gene (probe 1, Jones et al., 1994). The clones were characterized by restriction enzyme mapping and DNA gel blot analysis from which a physical map for each locus could be deduced (Figure 1). The sequence of all three haplotypes at the *Cf-4/9* locus was determined.

Both the *Cf-4* and the *Cf-9* genes are located within 33 kb and 36.5 kb regions, respectively, each containing four additional *Hcr9s*. *Hcr9s* were named according to the near isogenic line from which they originated and their relative position in the cluster; e.g., *Hcr9–9A* (shorthand *9A*) refers to the 5′-most gene in the *Cf-9* cluster and *Hcr9–4A* (*4A*) to the corresponding gene in the *Cf-4* cluster. All *Hcr9s* are transcribed in the same direction and have intronless open reading frames (ORFs). cDNAs have been isolated for five *Hcr9s* (data not shown), including *Cf-9*, and in all cases a single intron was identified in the 3′ untranslated region as described for *Cf-4* (Thomas et al., 1997). All *Hcr9s* at the *Cf-4/9* locus encode proteins with 27 LRRs with the exception of 4B, which contains 23 LRRs, and Cf-4, which comprises 25 LRRs.

One band hybridizing to the *Cf-9* probe 1 was identified in L. esculentum DNA that was linked in repulsion to *Cf-4* and *Cf-9* (see Experimental Procedures). This was present as a single band on a phage clone isolated from Cf0, indicating that this particular L. esculentum genotype carries only one *Hcr9* (*Hcr9–0*) at the corresponding locus.

Conserved Lipoxygenase Sequences Flank the Locus

In all three haplotypes analyzed, the *Cf-4/9* locus is flanked by two convergently oriented sequences highly homologous to a number of cDNAs encoding plant lipoxygenases (Figure 1). The highest degree of identity (>94%) was observed with a cDNA for *LoxC* from L. esculentum. The two lipoxygenase sequences delimit the 5′ (*LoxL*) and 3′ (*LoxR*) ends of the *Hcr9* clusters. The *LoxL* sequences from the three haplotypes are more closely related to each other than are the *LoxL* and *LoxR* genes. The intron sequences of *LoxL* and *LoxR* show no homology. The region carrying the *R* genes is characterized by excessive polymorphism between the haplotypes, whereas the flanking *Lox* sequences are highly conserved (Figures 2 and 3). DNA of plants recombinant between the RFLP markers CP46 and TG236 flanking *Cf-4/9* (Balint-Kurti et al., 1994; Thomas et al., 1995) was prepared from F2 populations of the crosses L. pennellii × Cf4 or Cf9 and subjected to DNA gel blot analysis with the *Cf-9* probe 1. Using this approach, no additional *Hcr9s* were identified that cosegregated with *Cf-4* or *Cf-9* (data not shown). The identification of conserved flanking sequences and the lack of additional cosegregating *Hcr9s* confirmed that the entire resistance gene locus had been cloned. The conserved *LoxL* and *LoxR* sequences probably represent the progenitor

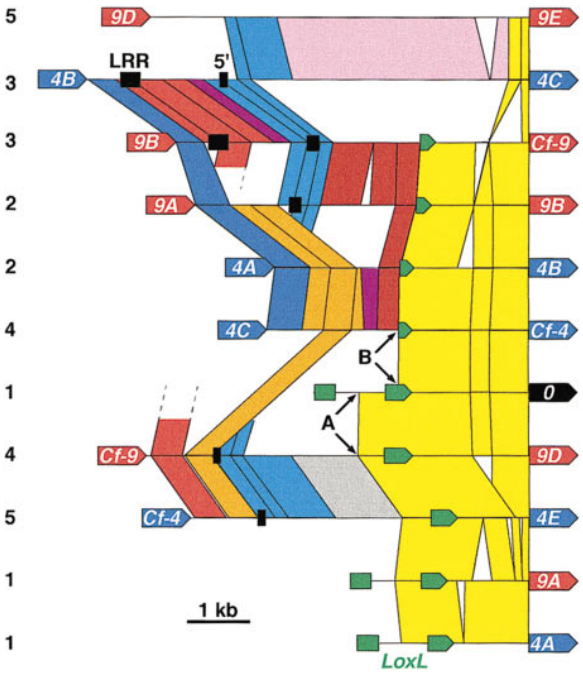


Figure 2. Schematic Comparison of the *Hcr9* Intergenic and 5′ Flanking Regions at the *Cf-4/9* Locus

The sequences have been ordered so that most homologous intergenic regions are adjacent. Their relative position in the clusters is indicated by numbers on the left, where position 1 corresponds to the 5′-most region. Sequences exhibiting more than 90% homology are shown in identical colors (some deletions <100 bp are not shown, but are accounted for by the difference in width of some colored bars). The ORFs of the *Hcr9s* are shown by filled arrows not drawn to scale. They are colored according to the line from which they were isolated (black, Cf0; blue, Cf4; red, Cf9). The exons of *LoxL* are indicated by green symbols. The *LoxL–Hcr9* stretch is shown in yellow. The position of the two proposed ancient recombination breakpoints is indicated by arrows (A and B). The position of truncated fragments of *Hcr9s* is indicated by black boxes. LRR: sequence with homology to region encoding LRR 20–22 of Cf-9, 5′: *Hcr9* fragment with homology to a region flanking the translational start site.

flanking sequence of the clusters. A hypothetical ancestor of the complex *Cf-4/9* locus most likely consisted of a single *Hcr9* gene flanked by *LoxL* and *LoxR* sequences. Although the Cf0 haplotype resembles this arrangement most closely, it is possible that this is the product of one or several unequal recombination events between tandemly repeated genes. In each of the clusters, four out of five *Hcr9* genes are flanked upstream by related stretches of DNA encompassing a truncated *LoxL* fragment, suggesting that this region was duplicated with an ancestral *Hcr9* during the formation of the tandemly repeated *Hcr9* cluster. Identical fragments are found in the *Cf-4* and the *Cf-9* clusters, indicating that these fragments evolved before speciation of L. hirsutum and L. pimpinellifolium. Two classes of *LoxL* fragments were found (Figures 1 and 2). The members of one class are present 5′ of *4E* and *9D* and contain the entire 3′-most exon of *LoxL* as well as 480 bp of the preceding intron. The sequence 5′ of these fragments is identical but differs from the *LoxL* progenitor sequence. The point of divergence therefore defines one ancient recombination breakpoint (arrow A in Figure 2). The other class is found in the 5′ regions of *Cf-4, Cf-9, 4B*, and *9B* and consists of 214 bp of a truncated 3′-most exon. The sequence immediately 5′ of these four short *LoxL* fragments diverges from *LoxL* but is identical between the four, defining a second ancient breakpoint (arrow B in Figure 2). From these observations it appears likely that the recombination breakpoint that led to the initial duplication of an *Hcr9* progenitor sequence occurred in the *LoxL* sequence.

Sequence Rearrangements in the Intergenic Regions

A detailed analysis of sequence relationships between all the intergenic regions in the cluster as well as the 5′ regions of *4A, 9A*, and *0* was performed (Figures 2 and 3). Pairwise diagonal dot plot comparisons revealed blocks of extensive sequence homology between most of the intergenic regions. However, the position and length of these blocks varied between pairwise comparisons, indicating sequence rearrangements. These rearrangements appear to have occurred within certain limits. First, apart from several inverted repeats smaller than 20 bp, larger inversions were not found. Second, the order of homologous blocks is never changed between different intergenic regions; i.e., ABC never becomes BCA. Therefore, several independent deletion events may have reduced an ancestral intergenic sequence to the variants observed now.

Within the *Cf-9* cluster, all the intergenic regions are distinct from each other with respect to their sequence block pattern (Figure 3). In the *Cf-4* cluster, the intergenic region between *4C* and *Cf-4* (*4C:Cf-4*) is nearly identical to *4A:4B*. As these sequences appear not to have experienced extensive rearrangements, they are possibly the product of a more recent duplication event. Intergenic regions of highest similarity between the *Cf-4* and *Cf-9* clusters suggest common ancestors but were found in nonallelic positions. This nonsyntenic arrangement of intergenic regions is exemplified by the pairs *Cf-9:9D/Cf-4:4E* (position 4 in the *Cf-9* cluster versus position 5 in the *Cf-4* cluster) and *4B:4C/9D:9E* (position 3 in the *Cf-4* cluster versus position 5 in the *Cf-9* cluster).

Two possible remnants of intragenic rearrangements were found. An ~200 bp fragment with strong homology to the region flanking the ATG of *Hcr9* genes is present three times in the *Cf-9* cluster and twice in the *Cf-4* cluster. Their similarity suggests that they are the products of duplication events. A second region present once in both clusters comprises a 177 bp stretch with homology to LRRs 20–22 followed by 511 bp with near identity to the 3′ nontranslated region of the *Cf-4* and *Cf-9* genes. The fact that these *Hcr9* fragments are nearly identical between the *Cf-4* and *Cf-9* clusters suggests they are the product of events that occurred before speciation. The limited representation of intragenic fragments and the lack of obvious pseudogenes of *Hcr9s* are in striking contrast to the high degree of sequence rearrangements in the intergenic regions, indicating a selection for maintenance of the ORFs in this region.

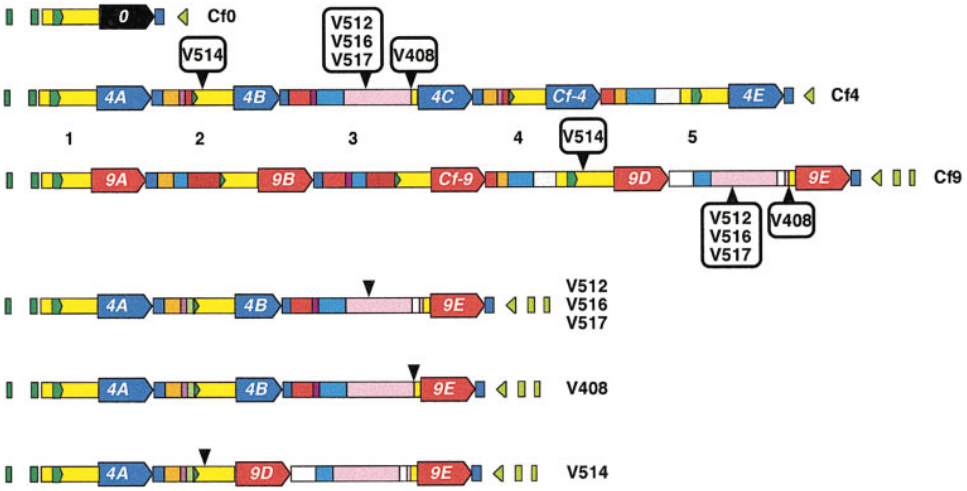


Figure 3. Sequence Relationships between Eight Allelic Clusters at the *Cf-4/9* Locus

Stretches of nearly identical sequences are shown in the same colors, except for the ORFs of *Hcr9s*, which are colored according to the lines from which they originated (black, Cf0; blue, Cf4; red, Cf9). The use of colors is consistent with Figure 2. Note the degree of polymorphism between the intergenic regions of each cluster and the nonsyntenic arrangement of intergenic regions exhibiting highest homology. The exons of lipoxygenase are shown by dark green arrows and boxes. The five chromosomes of the disease-sensitive Cf4/Cf9 recombinants V408, V512, V514, V516, and V517 are shown. The arrows indicate the position of the recombination breakpoints. The breakpoint in V514 was located between 1266 and 1395 bp upstream of the ATG of *9D*. In V516, V512, V517, and V408, the breakpoints were located in 22, 50, 88, and 76 bp intervals, which were 3373, 2905, 2817, and 419 bp upstream of the ATG of *9E*, respectively.

The Hcr9 Consensus Sequence Exhibits Seven Hypervariable Amino Acid Positions within a Background of High Overall Sequence Conservation

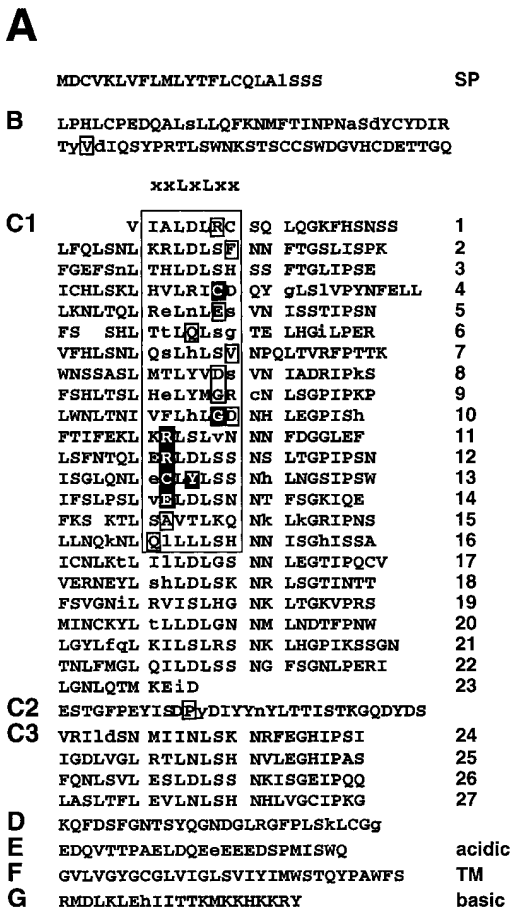
The *Hcr9* gene family exhibits a high degree of overall sequence homology. Sequence identities at the nucleotide level range from 92.19% for the pair *Cf-4/4C* to 99.34% for the pair *4C/9E*. At the amino acid sequence level, the identities ranged from 99.31% for the pair 4A/9A and 85.36% for 4B/Cf-9. Three *Hcr9s* also exhibit deletions of variable extent and position relative to the *Hcr9* consensus sequence in the 5′ portion of the gene. Hcr9–0 carries a deletion encompassing half of the predicted signal peptide and at least 7 amino acids of the B domain (The Hcr9 domain structure is depicted in Figure 4). This deletion could lead to aberrant processing of the gene product, which is consistent with the lack of resistance of Cf0 toward all tested races of C. fulvum.

In Cf-4 two LRRs are deleted and in 4B the second half of the B domain and the first 4.5 LRRs are deleted. Some smaller deletions of 1–10 amino acids are present in other Hcr9s that are not predicted to affect the structure of the proteins. All the deletions described maintain an intact ORF.

Within the high degree of overall similarity in Hcr9 proteins, conserved as well as hypervariable regions can be defined by examining the number of alternate amino acid residues at a given position. A consensus sequence generated by alignment of the 11 Hcr9s comprises 867 amino acids (Figure 4). At 806 positions (92.6%) a single or one of two amino acids were found. In 44 positions (5.1%) three different amino acids were observed. In 13 positions (1.5%) 4 amino acids (variable position) were found, and in only 7 (0.8%) positions 5 or more amino acids were found (hypervariable position). The majority of the variable and hypervariable positions are clustered within the interval between the second half of the B domain and LRR16. In general, each of the LRRs 1–16 carries a single variable or hypervariable position. Exceptions are LRR3, where no variable position was observed, and LRRs 10 and 13, which each have two variable positions. All of these positions are within a region of the LRR consensus sequence (XXLXLXX), which is predicted to form a β-strand/β-turn structure (Jones and Jones, 1997) as observed in the porcine ribonuclease inhibitor (Kobe and Deisenhofer, 1995), but do not include the conserved leucine residues (Figure 4). The leucine residues project into the hydrophobic core of the protein while the sidechains of the flanking amino acids are solvent exposed where they may interact with ligand molecules (Kobe and Deisenhofer, 1995). Sequence variability within the interstitial residues will affect ligand binding and may therefore confer distinct recognitional specificities to each Hcr9.

Enhanced Rate of Nonsynonymous Nucleotide Substitutions in the Solvent-Exposed Residues of the β-Strand/β-Turn Motif of the First 16 LRRs

A suitable method to determine the type of selection acting on a gene family is a comparison of synonymous (Ks) and nonsynonymous (Ka) substitution rates per synonymous/nonsynonymous site. With no selection pressure being present, the Ka/Ks ratio is predicted to be 1, a ratio that has been observed in pseudogenes (Hughes, 1995). At amino acid positions constituting the antigen recognition site of predicted MHC class I proteins, Hughes and Nei (1988) found Ka:Ks ratios $>$ 1, which



B

	synonymous	nonsynonymous
xx(L)x(L)xx LRR 1-16	19.8±7.2	26.2±6.6
nonframed	8.1±2.1	4.8±0.7

Figure 4. Distribution of Amino Acid Variability in Hcr9 Sequences

(A) Domain structure of Cf-9 according to Jones and Jones, 1997. SP, signal peptide; TM, transmembrane domain. LRRs 1–27 are indicated by numbers on the right. The position of variable amino acid residues (smallprint, 3; or boxed, 4 different amino acids found) and hypervariable (shaded, 5 or 6 different amino acids found) in peptide sequence alignments of Hcr9s are superimposed on the Cf-9 sequence. The predicted β-strand/β-turn structural motif of the LRRs is indicated by the position of "xxLxLxx" in a line above LRR 1. (B) Synonymous and nonsynonymous nucleotide substitutions in different regions of the *Hcr9* coding sequence. The rates of synonymous or nonsynonymous substitutions per synonymous/nonsynonymous site were calculated for 55 pairwise comparisons between the eleven *Hcr9* genes using NewDiverge. Files containing the concatemerized nucleotide sequences encoding the XX(L)X(L)XX of LRR1-16 were generated for each *Hcr9* (framed region in [A]). The conserved hydrophobic positions (L) were omitted as these residues serve a structural role and are probably subjected to selection for sequence conservation. The 11 sequences obtained in this way were aligned with each other and subjected to analysis by NewDiverge. Complementary sequence files of the total remainder of the coding region were generated, aligned, and analyzed by NewDiverge in which the nucleotide sequences encoding XXLXLXX of LRRs 1-16 were removed (nonframed).

they interpreted to be a consequence of diversifying selection. The rates of synonymous and nonsynonymous nucleotide substitutions between all *Hcr9s* were calculated for the XX(L)X(L)XX sequence of the first 16 LRRs. This region is highlighted by a frame in Figure 4, but the conserved hydrophobic leucine residues were omitted, as they serve a structural role (Jones and Jones, 1997). The average rate of nonsynonymous substitutions (Ka) in this region was more than five times higher than in the remainder of the protein (Figure 4) and the rate of synonymous substitutions (Ks) was more than two times higher. The fact that Ka and Ks are both higher in the XX(L)X(L)XX region of LRRs 1–16 indicates that this region is evolving more rapidly. Furthermore, a Ka:Ks ratio > 1 was found, indicating a positive selection for diversification has acted on the predicted β-strand/β-turn region of the first 16 LRRs.

Comparison of *Hcr9* Family Members Reveals a Patchwork of Sequence Similarities

Within nucleotide sequence alignments of the *Hcr9* coding regions, polymorphic sites could be detected. At informative polymorphic sites (IPS), 2 or more genes carry the same nucleotide. Using these IPS, sequence stretches shared by two or more members of the gene family have been identified. A clear sequence affiliation was inferred whenever three or more consecutive IPS were contained within two or more genes (sequence fingerprints; Figure 5). Apart from the gene pairs *4A/9A* and *4C/9E,* which exhibited almost continous sequence affiliations throughout their ORFs, sequence affiliations between all other gene pairs were restricted to shorter sequence stretches. The closest relative was found to vary according to the particular stretch of nucleotide sequence under inspection (Figure 5). The *Hcr9* gene family therefore represents a patchwork of sequence affiliations. This patchwork nature suggests that sequence exchange events between *Hcr9s* have been involved in the evolution of novel variants. Conceptually, exchange of sequence stretches between ORFs can result in novel codons at the breakpoints. We superimposed the codon positions of the seven hypervariable amino acids on the *Hcr9* patchwork structure. In at least two cases, the borders of fingerprints indicating a breakpoint are within such a codon (Figure 5). This result suggests that novelty in the hypervariable positions can at least partly be attributed to exchange events.

Similarities between the intergenic regions are in three cases reflected by sequence affiliations between flanking *Hcr9s*. The closely related *4A* and *9A* genes share the 5′ flanking position of the clusters and are preceded by the conserved *LoxL* sequence. The *4C* and *9E* genes exhibit uninterrupted sequence affiliation throughout their ORFs and reside in an 8 kb stretch of highly homologous DNA. Each of these pairs therefore shares a common ancestor and has not differentially recruited sequences from other *Hcr9s*. However, a significant divergence between *4A* and *9A* is observed at the 3′ end, indicating a possible recombination event with a yet unidentified *Hcr9*. The ORFs of *Cf-4* and *Cf-9* genes only differ by one nucleotide in 1057 bp at their 3′ ends (Thomas et al., 1997). This sequence similarity extends

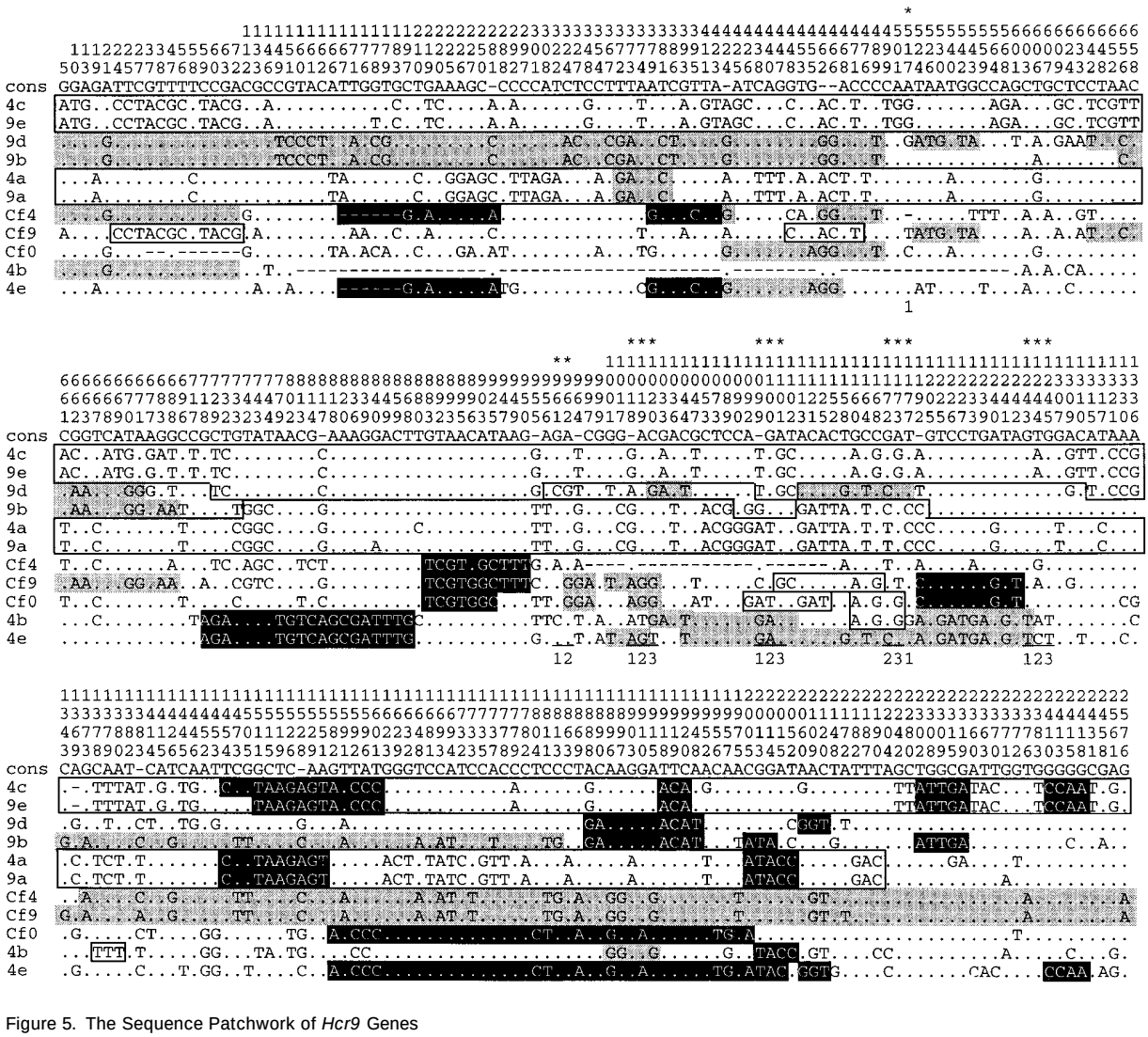


Figure 5. The Sequence Patchwork of *Hcr9* Genes

Only informative polymorphic sites of the nucleotide sequences of the 11 *Hcr9s* are shown. This reduces the total length of the sequence alignment from the 2601 consensus residues to 302. The vertical numbers at the top of each row indicate the corresponding nucleotide position in the full consensus sequence relative to the first base in the ATG translational start codon. The almost continous sequence affiliations within the gene pairs *4A/9A* and *4C/9E* are both boxed by lines, as are some patches of sequence identity between one of these pairs and other *Hcr9s*. Smaller boxes of identical color indicate uninterrupted sequence affiliations. The nucleotides under an asterix are part of the codons encoding the seven hypervariable amino acid positions. The number below these positions indicates the nucleotide position within the codon. Two possible recombination breakpoints within these codons are found between nucleotide position 1019 and 1020 of the *4E*, *Cf-9*, and *9D* genes and between 1243 and 1244 of the *4B* and *4E* genes. cons, consensus sequence; (dots) nucleotides identical to the consensus; (dashes) gaps in the alignment.

for a further 5.2 kb beyond the stop codon, again suggesting a common ancestor. The 5′ flanking intergenic region of the *Cf-4* gene is nearly identical to that of *4B*, a relationship that continues into a shared fingerprint at their 5′ ends (Figure 5). In contrast, the *Cf-9* gene exhibits a clear sequence affiliation with *4C* and *9E* at its 5′ end and the flanking intergenic region is most similar to that of *9B*. This flanking marker exchange between the *4B*, *Cf-4*, *9B*, and *Cf-9* genes indicates the involvement of intragenic recombination events in their evolution. In other cases, remarkably little correlation between sequence relationships in the intergenic regions and their flanking *Hcr9s* was observed. This might be a reflection of the relatively high rates at which sequence exchange between *Hcr9s* has occurred.

Meiotic Stability of *Cf-4* and *Cf-9*

The meiotic stability of *Cf-4* and *Cf-9* was determined by screening for loss of *Cf-4* or *Cf-9* gene function in testcross progeny of Cf9 plants (homozygous for the *Cf-9* haplotype) and in testcross progeny of a *Cf-4/9* transheterozygous stock. In a homozygous background, the *Cf-9* gene was found to be very stable. No surviving plants were identified in 12,000 testcross progeny between Cf9 and a transgenic plant homozygous for expression of the avirulence determinant Avr9. Progeny

from this cross that carry both *Cf-9* and *Avr9* die at the seedling stage (Jones et al., 1994), providing a sensitive assay for *Cf-9* function. Consistent with this result, no disease-sensitive individuals were recovered from more than 10,000 Cf9 × Cf0 testcross progeny inoculated at the cotyledon stage with C. fulvum race 5 (M. Dixon, personal communication).

Recombination between Nonsyntenic Stretches of Homology Resulted in Disease-Sensitive Progeny

In comparison to the *Cf-9* homozygote, the meiotic stability of *Cf-9* was dramatically reduced in a *Cf-4/9* transheterozygous background. Five disease-sensitive individuals were identified when 7,500 testcross progeny of a *Cf-4/9* transheterozygous cross to Cf0 were inoculated with C. fulvum race 5 (which expresses *Avr4* and *Avr9*). In all five recombinant chromosomes, the number of *Hcr9s* was reduced and both the *Cf-4* and *Cf-9* genes were absent. In the recombinant V514 only *4A, 9D*, and *9E* were present (Thomas et al., 1997). The recombination breakpoint was mapped within the *4A:4B* and *Cf-9:9D* intervals. Sequence comparison of these intervals revealed a 2.2 kb stretch of near sequence identity that could serve as a template for unequal pairing and recombination. In the recombinants V512, V516, and V517, bands diagnostic for *4A, 4B*, and *9E* could be detected (Thomas et al., 1997). The most likely recombination templates in these recombinants were the flanking regions and ORFs of *4C* and *9E*, where alignments revealed an 8 kb stretch of highly homologous sequence. Fragments spanning the delimited regions were amplified by PCR and sequenced. Two nucleotide positions polymorphic between the two parental sequences revealed the positions of the breakpoints. All the breakpoints are located within nonsyntenic stretches of high DNA sequence homology (Figure 3). In each recombinant, an unequal crossover resulted in two recombinant chromosomes that differ in *Hcr9* number from the parental clusters. For each of the recombinant chromosomes lacking *Cf-4* and *Cf-9*, there should have been a counterpart which carried both genes. The latter recombinants were not identified in our screen.

Figure 6. Resistance Phenotypes Conferred by *Hcr9* Genes

Disease symptoms on Cf0 plants transformed with either cosmid 2/9-75 (*9A* and *9B*, see Figure 1) or cosmid 2/9-45 (*9E*) 21 days postinoculation with C. fulvum race 5 (*Avr4*, *Avr9*). All plants were inoculated at the eight-leaf stage. Cosmid 2/9-75 confers resistance associated with extensive necrosis and chlorosis (category Rn, Table 1), while cosmid 2/9-45 mediates a delayed and reduced sporulation phenotype (category dS, Table 1). In contrast, the Cf0 untransformed control (Cf0) allows abundant fungal sporulation over the entire lower leaf surface, whereas the Cf9 control (Cf9) is fully resistant. All photographs were taken under a polarizing light.

Hcr9s Additional to *Cf-4* and *Cf-9* Specify C. fulvum Resistance in Adult Plants

When the Cf9 line was inoculated at the eight-leaf stage, it was resistant to C. fulvum race 5.9, which lacks *Avr9* (Marmeisse et al., 1993). Cf0 is susceptible to this race. This result suggests that the introgressed DNA from L. pimpinellifolium, which carries *Cf-9*, contains an additional gene(s) that confer resistance to C. fulvum. We tested other *Hcr9s* in the *Cf-9* cluster for their ability to confer adult plant resistance. On transformation into Cf0 plants, at least two other *Hcr9* genes were shown to inhibit the growth of C. fulvum after inoculation of either primary transformants at the eight-leaf stage or T2 progeny at the six-leaf stage and older. Cf0 plants harboring cosmid 2/9–75 (Figure 1) carrying both *9A* and *9B* developed necrotic and chlorotic symptoms on inoculated leaves (Figure 6). Limited fungal growth was detected but sporulation was never observed. Either *9A* or *9B* or both are responsible for this resistance response. Cosmid 2/9–45 carrying *9E* on its own conferred a delay of C. fulvum race 5 sporulation by one week compared to the fully susceptible cultivar Cf0 (Figure 6). All transgenic lines, when inoculated at either the cotyledon or three-leaf stage, were fully susceptible to C. fulvum race 4,

Table 1. Responses of Tomato Transformants Carrying Different *Hcr9-9* Genes to Three Different Races of Cladosporium fulvum

		C. fulvum Race Inoculated[a]											
		Race 5 (avr4$^+$, avr9$^+$)				Race 5.9 (avr4$^+$, avr9$^-$)				Race 4 (avr4$^-$, avr9$^+$)			
	Number of Plants/Lines[c]	R	Rn	dS	S	R	Rn	dS	S	R	Rn	dS	S
Controls													
Cf9	12/1	12	0	0	0	12	0	0	0	12	0	0	0
Cf0	12/1	0	0	0	12	0	0	0	12	0	0	0	12
Cf4	12/1	12	0	0	0	12	0	0	0	0	0	0	12
Cosmid 9-75 (Hcr9-9A and B)													
Primary transformants	1/12	0	9	0	3			ND				ND	
T_2 progeny													
Line B	18/1	0	5	0	1[b]	0	6	0	0	0	4	0	2[b]
Line E	20/1	0	6	0	0	0	5	0	1[b]	0	7	0	1[b]
Line I	19/1	0	4	0	2[b]	0	5	0	0	0	6	0	2[b]
Line K	20/1	0	7	0	1[b]	0	6	0	1[b]	0	5	0	0
Line L	19/1	0	5	0	1[b]	0	5	0	1[b]	0	6	0	1[b]
Cosmid 9-45 (Hcr9-9E)													
Primary transformants	1/5	0	0	3	2			ND				ND	
T_2 progeny													
Line A	17/1	0	0	5	1[b]	0	0	6	0	0	0	4	1[b]
Line B	19/1	0	0	5	0	0	0	4	1[b]	0	0	7	2[b]
Line C	20/1	0	0	6	1[b]	0	0	5	0	0	0	6	2[b]

R, fully resistant; Rn, resistant with visible necrosis and chlorosis; dS, delayed sporulation (day 21+); S, wild-type sporulation (day 14); ND, not determined.

[a] Tomato plants inoculated at the 8-leaf growth stage.

[b] NPT assay negative, indicating these T_2 plants had not inherited the T-DNA. The NPT$^-$ sibling plants were as susceptible to C.fulvum as the Cf0 controls.

[c] Number of plants per independent genotype (line) inoculated.

race 5, and race 5.9, so the resistance responses mediated by *9E* and *9A* or *9B* are only observed in adult plants. The resistance phenotypes of Cf4/Cf9 recombinants V408, V516, V517, and V514 were indistinguishable from the one observed with Cf0 plants transgenic for *9E*. As the sporulation of race 4 on adult Cf4 plants was identical to that on Cf0 plants, either *9E* or other genes located distal to the *Cf-9* cluster are responsible for the delayed sporulation of race 4 on older Cf4/Cf9 recombinant plants. When Cf0 plants expressing *Hcr9s* transgenically were inoculated with either race 5.9 or race 4 (mutant in Avr4; Joosten et al., 1994), both races provoked identical resistance phenotypes as race 5, which produces both Avr4 and Avr9 (Table 1). These data indicate that Avr4 and Avr9 are not necessary for the onset of the *Hcr9*-mediated resistance phenotypes. Consequently, another avirulence specificity must be recognized by either *9A* or *9B*. *9E* may recognize an additional distinct avirulence determinant.

Discussion

The *Cf-4/9* Locus Encodes Multiple Resistance Specificities

Resistance specificities and *R* gene homologs are frequently clustered in plants. In Arabidopsis, soybean, and potato, *R* gene homologs have been shown to be genetically linked to *R* gene specificities (Kanazin et al., 1996; Leister et al., 1996; Botella et al., 1997). The *N* gene of tobacco is a member of a genetically linked gene family (Whitham et al., 1994), and some *Xa21* gene family members of rice are physically linked in tandem repeats (Song et al., 1997). Multiple homologs of the *L6* gene reside at the *M* locus in flax, one of which is the *M* gene (Anderson et al., 1997). The *Pto* and *Fen* genes of tomato discriminate between the products of the bacterial *avrPto* gene and the insecticide fenthion and belong to a gene family of serine/threonine protein kinases (Loh and Martin, 1995), members of which are physically linked (Salmeron et al., 1996). Two tandemly repeated copies of the *Cf-2* genes are physically linked to additional *Hcr2s* (Dixon et al., 1996). Based on these and related findings it has been speculated that clustered *R* gene specificities might be caused by clustered *R* gene families. Here we show that tandemly repeated functional *R* gene homologs at the *Cf-4/9* locus are indeed responsible for clustered specificities. Four *Hcr9* genes (*Cf-4*, *Cf-9*, *9E*, and *9A* or *9B*), the products of which discriminate at least three different *Avr* genes of C. fulvum, are found at the *Cf-4/9* cluster.

The *Cf-9* locus confers durable resistance that has not been overcome since its introgression into commercial cultivars. This durability is probably due to additional *R* gene specificities linked to *Cf-9*. The absence of pseudogenes and the presence of three distinct specificities implies that strong selection pressure may have been imposed on the *Hcr9* gene family by the ability of the fungus to evade recognition by individual *Hcr9s*. Races that overcome the *Cf-9*- or *Cf-4*-mediated seedling resistance have lost *Avr9* (Van den Ackerveken et al., 1992) or carry mutant versions of *Avr4* (Joosten et al., 1994). Durable resistance might be reinforced by differential expression of *R* gene specificities during development, which could be regarded as natural pyramiding of *R* genes. The *Cf-4* and *Cf-9* genes are active from the seedling stage onward. However, the effects of *Hcr9s*

9E and *9A* or *9B* are only detectable in adult plants. Conceptually, this transient susceptibility provides temporal refugia for races carrying *Avr* genes that compete with eventually emerging supervirulent races. By this mechanism, their spread could be slowed down.

Definition of Functional Domains

Hcr9 gene products are predicted to have two functions. First, recognition of specific *Avr* gene products requires sequence variability at amino acid positions that contribute to recognitional specificity. Second, they are thought to signal through a common transduction pathway to induce defense responses, which requires sequence conservation in those parts of the Hcr9 that might interact with a partner molecule to initiate a signal transduction cascade. Cf-4 and Cf-9 have identical C termini, and the same region shows homology even to the product of the unlinked *Cf-2* gene (Dixon et al., 1996). The specificity of recognition must therefore reside in the N-terminal portion, where a significant degree of sequence divergence was found between Cf-4 and Cf-9 (Thomas et al., 1997). The comparison of 11 Hcr9 sequences allowed us to delimit a possible recognition domain even further. In contrast to the remainder of the protein, which exhibits extensive sequence homology, we observed a high degree of variability in the interstitial amino acid residues of the β-strand/β-turn region that are predicted to be solvent exposed, based on the structure of the porcine ribonuclease inhibitor (Kobe and Deisenhofer, 1995). The high degree of polymorphism of these residues as well as their predicted solvent-exposed position suggests an involvement of these amino acids in ligand binding. This model is further supported by the finding that diversifying selection is acting on this domain. In the presence of pathogen diversity, multiple variants of *R* genes potentially conferring different recognition specificities are expected to be beneficial. Here we present evidence that sequence diversity is positively selected for in a region predicted to interact directly with a ligand. In contrast, the remainder of the protein exhibits a Ka/Ks ratio smaller than 1 indicative of selection for amino acid sequence conservation. Structural restrictions within the LRRs and the need for interaction with a common downstream signaling partner (Dixon et al., 1996; Thomas et al., 1997) might impose conservative selection pressure.

Novel *Hcr9* Variants Are Generated by Sequence Exchange between Gene Family Members

The patchwork pattern of sequence fingerprints shared between *Hcr9s* is clear evidence for sequence exchange between them. A high proportion of the overall polymorphism between the genes can be attributed to the shuffling of sequence patches, which we therefore propose to be a major mechanism generating novel *Hcr9* genes. Both the MHC class II of mammals (Andersson and Mikko, 1995) and the tomato *Hcr9* loci are comprised of patchwork genes, indicating similar modes of evolution of novel recognition specificities. The observed sequence patches could result either from successive rounds of reciprocal recombination or from gene conversion events. In the *bronze* gene of maize, as little as 19 bp of uninterrupted sequence identity between polymorphic nucleotides is sufficient for the resolution of recombination intermediates (Dooner and Martínez-Férez, 1997). Likewise, our evidence for recombination between polymorphic *Hcr9* genes suggests that long stretches of identity are not required for the resolution of recombination intermediates in tomato.

Tandemly repeated gene families have been observed to exchange sequences rapidly thereby generating homogeneity between their members, a phenomenon called concerted evolution (Dover et al., 1993). In humans, the snRNA genes are arranged in short clusters of four to five tandem repeats. This gene family is subjected to concerted evolution, as all of the members of one haplotype share identical sequences (Liao et al., 1997). In the rDNA cluster of yeast, gene conversion between sister chromatids has been found to be the major force leading to sequence homogeneity (Gangloff et al., 1996). Thus, our finding of sequence exchange between *Hcr9* genes is probably a reflection of a more general mechanism directing the evolution of tandemly repeated genes.

Polymorphic Intergenic Regions Determine the Frequencies of Recombination between Haplotypes

We found clear evidence for sequence exchange between Hcr9s. If unchecked this could lead to complete sequence homogenization. How then can variability, an indispensable source of novel resistance specificities, be achieved and maintained in the *Hcr9* gene family? We do observe a significant degree of sequence variability between the *Hcr9* genes in a single haplotype at the *Cf-4/9* locus. This means that concerted evolution at this locus is limited so that *R* gene variants can both arise and persist. Sequence rearrangements have previously been shown to be associated with suppression of recombination at the mating type locus of the green alga Chlamydomonas reinhardtii (Ferris and Goodenough, 1994) and the S locus of Brassica species (Boyes et al., 1997). Likewise, the sequence rearrangements observed in intergenic regions within the *Cf-9* locus are associated with low frequencies of spontaneous loss of *Cf-9* function. Unequal alignment between different genes of the *Cf-9* cluster could result in loss of *Cf-9* by gene conversion or recombination. The fact that we do not observe such events is probably a consequence of suppression of mispairing. This suppression could be mediated by the polymorphism of the intergenic regions, which would lead to improper sequence alignments in a nonallelic situation (Figure 7). Apparently, the homology between the ORFs and the related *LoxL-Hcr9* regions, which are present 4 times in each cluster (yellow regions in Figures 2 and 3), are not sufficient to compensate for this. The latter regions are distinguished by point mutations and differential deletions smaller than 806 bp. Insertions and point mutations are associated with reduced recombination frequencies in the *bronze* gene of maize (Dooner and Martínez-Férez, 1997).

In strong contrast to the meiotic stability of the *Cf-9* gene in a homozygous background, we observe the loss of both resistance genes at a frequency of approximately 10^{-3} in testcross progeny of *Cf-4/9* transheterozygous plants (Thomas et al., 1997). Our analysis of

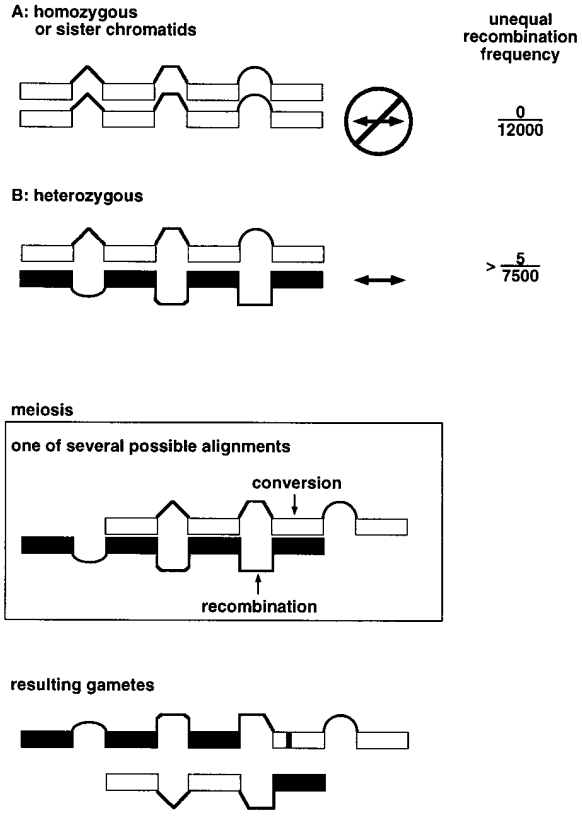


Figure 7. The Influence of Sequence Polymorphism in the Intergenic Regions on the Outcome of Haplotype Interactions

(A) In homozygotes as well as between sister chromatids, homolog slippage is suppressed due to the sequence differences between intergenic regions that only allow alignments between alleles. The observed frequency of *Cf-9* loss is very low, referring to survival rates in a testcross of a Cf9 to a Cf0 plant transgenic for the fungal *Avr9* gene.

(B) In heterozygotes, proper sequence alignments are not possible between allelic intergenic regions. At meiosis, an increased frequency of nonreciprocal recombination events is observed. Recombination rates refer to the events analyzed between the *Cf-4* and *Cf-9* clusters. The occurrence of intragenic recombination or conversion events is suggested by the patchwork nature of the gene family. The resulting gametes have not only altered *Hcr9* numbers but also altered *Hcr9* combinations within each resulting haplotype.

these Cf4/Cf9 recombinants suggests that recombination is again determined by the specific arrangement of polymorphic intergenic regions of the parental clusters. In *Cf-4/9* transheterozygous plants, allelic *Hcr9s* are mostly preceded by dissimilar intergenic regions between which no perfect alignment can be achieved (Figure 3). However, several unequally positioned pairs provide potential templates for recombination due to the homologies that they share. We found that such nonsyntenically positioned stretches of homology are indeed used as recombination templates.

We therefore propose that the polymorphism of the intergenic regions serves a dual function. On the one hand, it suppresses unequal recombination in homozygotes and between sister chromatids, thereby preventing sequence homogenization of the gene family. On the other hand, between suitable heteroallelic haplotypes unequal alignment of homologous sequences and recombination is permitted. Such unequal recombination alters the *Hcr9* number and composition of the clusters, increasing the haplotype variation in the population. This model implies that the degree of haplotype diversity could determine the rate of generation of novel *R* gene loci, a concept supported by genetic analysis of the *Rp1* locus in maize (Sudupak et al., 1993). Exploiting those haplotype interactions might improve *R* gene management in breeding programs.

Experimental Procedures

Plant Transformation and Pathogenicity Tests

Plant transformation and growth conditions were as described previously (Jones et al., 1994). Plants were inoculated with C. fulvum race 5 (obtained from R. Oliver, Carlsberg Laboratory, Denmark) transgenic race 5.9 (Marmeisse et al., 1993), or race 4 GUS (Roberts et al., 1989) in a greenhouse (Hammond-Kosack and Jones, 1994). At the time of inoculation, plants were either at the seedling stage with fully expanded cotyledons and the first leaf was less than 5 mm in length (12–14 days after seed sowing), at the three-leaf stage (5 weeks after sowing), or at the eight fully expanded leaves stage (10 weeks after seed sowing).

Mapping and Isolation of the Cf0 Haplotype of the *Cf-4/9* Locus

F_2 populations of crosses between Cf4 or Cf9 to the disease-susceptible line FT33 of L. esculentum (Jones et al., 1994) were screened for recombination events between the markers FT33 and CP46 flanking the *Cf-9* locus. The line FT33 harbors a T-DNA approximately 2.2 cM proximal to *Cf-4/9* and constitutively expresses the *uidA* gene (GUS). F_2 progeny not expressing GUS (i.e., homozygous Cf9 or Cf4 at FT33) were screened for heterozygosity at the distal marker CP46 using CAPS or DNA gel blot analysis (Balint-Kurti et al., 1994). Seven recombinants were isolated from 560 Cf4 $\times$ FT33 F_2 and 4 recombinants from 331 Cf9 $\times$ FT33 F_2. DNA gel blot analysis of these recombinants revealed a 7.5 kb BamHI fragment hybridizing to the *Cf-9* probe 1 (Jones et al., 1994) to be absolutely linked in repulsion to *Cf-4* or *Cf-9*, respectively (see text). A phage library of partially MboI digested genomic L. esculentum Cf0 DNA was constructed in the vector EMBL3 (Stratagene) from which the corresponding clone was isolated. The sequence of *Hcr9–0* and its flanking DNA was established using a primer-walking strategy on subclones.

Sequence Analysis

Genomic libraries from Cf2/Cf9 and Cf4/Cf5 lines (Dixon et al., 1996; Thomas et al., 1997) were screened for *Hcr9* containing cosmids using *Cf-9* probe 1 (Jones et al., 1994). Cosmid 2/9–114, two XbaI subclones of cosmid 2/9–75, and a PstI subclone of cosmid 2/9–126 (Figure 1) were sheared, shotgun subcloned in pUC119, and sequenced as described (Dixon et al., 1996). The 3′ end of the *Cf-4* cluster which was not represented in cosmid clones was completed by sequencing PCR-amplified products with primers based on the *4E* sequence in combination with primers of the *LoxR* genes from the *Cf-9* locus. The specificity of these products was confirmed using genomic DNA from Cf0 and Cf9 as control templates.

Individual sequencing reads were assembled using the Staden package (Staden, 1996). Sequences were aligned and analyzed using Compare, Gap, Pileup, and NewDiverge (Wisconsin Package, Version 8, September 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, 53711 USA). Alignments were optimized using SeaView by Galtier et al., 1996, and informative polymorphic sites were displayed using Sequence Output (B. G. Spratt, University of Sussex, Brighton, UK).

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EMBL Accession Numbers

The EMBL nucleotide sequence database accession numbers of the L. hirsutum, L. pimpinellifolium, and L. esculentum haplotypes of the *Cf-4/9* locus are AJ002235, AJ002236, and AJ002237.