

Genetic Variation at the Tomato *Cf4/Cf9* Locus Induced by EMS Mutagenesis and Intralocus Recombination

Brande B. H. Wulff^{*,1} Colwyn M. Thomas^{†,1} Martin Parniske^{*} and Jonathan D. G. Jones^{*,2}

^{*}Sainsbury Laboratory, John Innes Centre, Norwich, NR4 7UH, United Kingdom and [†]School of Biological Sciences, University of East Anglia, Norwich, NR4 7TJ, United Kingdom

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ABSTRACT

The interaction between tomato (*Lycopersicon esculentum*) and the leaf mold pathogen *Cladosporium fulvum* is an excellent model for investigating disease resistance gene evolution. The interaction is controlled in a gene-for-gene manner by *Cf* genes that encode type I transmembrane extracellular leucine-rich repeat glycoproteins that recognize their cognate fungal avirulence (Avr) proteins. *Cf4* from *L. hirsutum* and *Cf9* from *L. pimpinellifolium* are located at the same locus on the short arm of tomato chromosome 1 in an array of five paralogs. Molecular analysis has shown that one mechanism for generating sequence variation in *Cf* genes is intragenic sequence exchange through unequal crossing over or gene conversion. To investigate this we used a facile genetic selection to identify novel haplotypes in the progeny of *Cf4/Cf9* trans-heterozygotes that lacked *Cf4* and *Cf9*. This selection is based on the ability of *Avr4* and *Avr9* to induce *Cf4*- or *Cf9*-dependent seedling death. The crossovers were localized to the same intergenic region defining a recombination hotspot in this cross. As part of a structure-function analysis of *Cf9* and *Cf4*, nine EMS-induced mutant alleles have been characterized. Most mutations result in single-amino-acid substitutions in their C terminus at residues that are conserved in other *Cf* proteins.

TOMATO *Cf* genes confer resistance to the leaf mold pathogen *Cladosporium fulvum* through recognition of distinct fungal-encoded avirulence (Avr) peptides secreted into the leaf apoplast during infection. The interaction between tomato and *C. fulvum* is an attractive model system to study the molecular basis of recognition specificity in plant disease resistance (R) proteins (VAN DER HOORN *et al.* 2001a; WULFF *et al.* 2001), R-protein-mediated defense responses (RIVAS and THOMAS 2002), and R gene evolution (PARNISKE *et al.* 1997; VAN DER HOORN *et al.* 2001b). *Cf* genes encode type I transmembrane glycoproteins composed predominantly of extracytoplasmic leucine-rich repeats (LRRs), a membrane-spanning region, and a short cytoplasmic domain that lacks an obvious signaling function (RIVAS and THOMAS 2002). *Cf* proteins, in common with many R proteins, activate a hypersensitive cell death response (HR) upon recognition of their cognate Avr proteins.

The tomato *Cf4* and *Cf9* genes were introgressed into the cultivated tomato *Lycopersicon esculentum* cv. "MoneyMaker" (*Cf0*), from wild relatives. Their protein products activate a plant defense response upon recognition of the *C. fulvum* Avr4 and Avr9 proteins, respectively. *Cf4* (from *L. hirsutum*) and *Cf9* (from *L. pimpinellifolium*) each reside in an array of five paralogs at the

Milky Way (*MW*) locus on the short arm of chromosome 1 (PARNISKE *et al.* 1997; THOMAS *et al.* 1997). Sequence analysis of the *Cf4* and *Cf9* haplotypes, together with the corresponding locus from the disease-sensitive *Cf0* line (PARNISKE *et al.* 1997), revealed extensive polymorphism within *Hcr9*'s (*H*omologues of *C. fulvum* resistance gene *9*). It was concluded that extensive intergenic and intragenic sequence exchange has occurred between paralogs. Selection for nonsynonymous nucleotide substitutions in sequences encoding the putative solvent-exposed residues of a conserved LRR structural motif also contributes to *Hcr9* sequence variation (PARNISKE *et al.* 1997). Other comparisons of R gene families have also revealed a higher rate of nonsynonymous to synonymous substitution within the putative solvent-exposed residues of the β -strand/ β -turn structural motif of LRRs (MCDOWELL *et al.* 1998; MEYERS *et al.* 1998; ELLIS *et al.* 1999; NOËL *et al.* 1999). This observation is consistent with the proposed role for LRRs in conferring AVR recognition specificity (DANGL and JONES 2001).

As a result of studies on several R gene families in Arabidopsis, flax, maize, rice, lettuce, and tomato, two models for R gene evolution have been proposed. On the basis of molecular analysis of the tomato *Pto* and the lettuce *Dm3* loci, MICHELMORE and MEYERS (1998) proposed the "birth and death" model, similar to the evolution of MHC alleles in mammals. In this model, R genes are "born" through duplication events, evolve in isolation, and "die" due to deletion or mutation. Sequence exchange is proposed to occur between orthologs, but is rare between paralogs, and point muta-

¹These authors contributed equally to this work.

²Corresponding author: Sainsbury Laboratory, John Innes Centre, Norwich, NR4 7UH, United Kingdom.
E-mail: jonathan.jones@sainsbury-laboratory.ac.uk

tion acting in concert with divergent selection appears to be the primary process generating novelty. The analysis of other *R* gene loci resulted in the so-called "permutation model" for *R* gene evolution (DODDS *et al.* 2001a), in which sequence exchange occurs between paralogs as well as orthologs. This model was deduced from analysis of the Arabidopsis *RPP5*, maize *Rp1*, flax *N* and *P*, and tomato *Cf-4/Cf-9* loci (PARNISKE *et al.* 1997; NOËL *et al.* 1999; DODDS *et al.* 2001a,b; SUN *et al.* 2001).

Comparative sequencing and the analysis of genetic stability at *R* gene loci have revealed key molecular mechanisms affecting *R* gene evolution such as inter- and intragenic recombination, gene conversion, mutation, and transposon insertion (RICHTER *et al.* 1995; PARNISKE *et al.* 1997; LUCK *et al.* 1998, 2000; COLLINS *et al.* 1999; NOËL *et al.* 1999; CHIN *et al.* 2001; SUN *et al.* 2001). The analysis of *Cf-4/Cf-9* recombinants could provide useful insights into gene evolution at this locus. Recombinants lacking *Cf-4* and *Cf-9* were originally identified in a genetic screen after inoculation with *C. fulvum* race 5 (that expresses *Avr4* and *Avr9*). However, a number of additional *Hcr9*'s have since been shown to confer resistance through recognition of different Avr determinants (PARNISKE *et al.* 1997; LAUGÉ *et al.* 1998a; TAKKEN *et al.* 1999; PANTER *et al.* 2002), and it is possible that some unequal crossovers would not have been recovered. In an attempt to overcome these limitations we developed a facile genetic selection to identify recombinants that lack *Cf-4* and *Cf-9*. This selection is based on the ability of *Avr4* and *Avr9* to induce *Cf-4* and *Cf-9*-dependent seedling death (HAMMOND-KOSACK *et al.* 1994a; THOMAS *et al.* 1997). As part of a structure-function analysis of Cf proteins, we have also characterized eight loss-of-function ethyl methanesulfonate-induced mutant alleles of *Cf-9* and one of *Cf-4*.

MATERIALS AND METHODS

Details of tomato lines used: Near-isogenic lines (NILs) of *L. esculentum* cv. Moneymaker containing *Cf-4* (Cf4), *Cf-9* (Cf9), or no genes for resistance to *C. fulvum* (Cf0) were maintained as described previously (DIXON *et al.* 1996, 2000).

Mutagenesis with EMS: The mutagenesis procedure was similar to that described previously (HAMMOND-KOSACK *et al.* 1994b). Approximately 3000 Cf9 seeds and 1000 Cf4 seeds were imbibed for 24 hr at room temperature in water. The seeds were incubated in a solution containing 60 mM EMS for 24 hr at 22°. After extensive washing the seeds were sown in batches of ~200 in seed flats. Viable seedlings were transferred to individual pots and treated with a 10⁻⁵ M solution of the gibberellic acid biosynthesis inhibitor paclobutrazol. This helped to maintain the large number of M₁ plants in a semi-dwarf state. The first fruit truss was removed since it is more likely to contain chimeric material. Selfed seed were collected from the second fruit truss of M₁ plants. The effectiveness of the mutagenic procedure was assessed by scoring several phenotypic characters in M₂ families as described by HAMMOND-KOSACK *et al.* (1994b). The frequency of visible mutations was similar to that reported previously (results not shown).

Mutant identification: Mutants compromised in their ability to induce an *Avr4*- or *Avr9*-dependent hypersensitive response were identified by inoculating M₂ families with recombinant potato virus X (PVX) expressing the *C. fulvum Avr4* (PVX:*Avr4*) or *Avr9* (PVX:*Avr9*) genes fused to the plant Pr1A signal peptide sequence for targeting to the leaf apoplast. In contrast to a previous report (THOMAS *et al.* 2000) that involved inoculating recombinant virus particles passaged through *Nicotiana clevelandii*, recombinant PVX was cloned into the T-DNA vector pGREEN (HELLENS *et al.* 2000) under control of the cauliflower mosaic virus 35S promoter. Recombinant clones were electroporated into *Agrobacterium tumefaciens* strain GV3101 containing the helper plasmid pSOUP. Stationary phase cultures were resuspended at an OD₆₀₀ of 0.5 in Murashige and Skoog salts containing 2-[*N*-Morpholino]ethanesulfonic acid (MES) adjusted to pH 5.6 with KOH, 2% w/v sucrose, and 10 μM acetosyringone. After 3 hr at room temperature bacterial suspensions were infiltrated into the cotyledons of M₂ seedlings. In Cf4 and Cf9 control plants a systemic Avr-dependent hypersensitive response could be observed after 7 days.

Kanamycin resistance assays: Resistance to kanamycin in tomato seedlings was determined by germination on Murashige and Skoog media supplemented with 300 μg ml⁻¹ kanamycin.

Generation of a line expressing *Avr4* and *Avr9*: A line expressing *Avr4* and *Avr9* transgenes was constructed by intercrossing a kanamycin-resistant transgenic line expressing *Avr9* (SLJ6021 A; HAMMOND-KOSACK *et al.* 1994b) with four independent kanamycin-resistant transgenic lines expressing *Avr4* (Avr4 7291 N, J, L, and M; THOMAS *et al.* 1997). The F₁ plants were self-pollinated and the segregation for resistance to kanamycin was tested in F₂ progeny. The ratio of kanamycin-resistant to -sensitive progeny in the cross SLJ6021 A × Avr47291 J was close to the predicted 15:1 ratio for independently assorting genes, and these progeny were analyzed further. PCR analysis was used to identify kanamycin-resistant plants containing *Avr4* and *Avr9*. Twenty-four individuals were then analyzed by DNA gel blots, using probes to the *Avr4* and *Avr9* transgenes and an endogenous tomato gene. Putative homozygotes for the *Avr4* and *Avr9* transgenes were identified on the basis of their relative hybridization intensity on blots. The genotypes of three individuals were confirmed in testcrosses to Cf4 and Cf9 plants. One line, designated Cf0 *Avr4*, *Avr9*, was used in testcrosses to identify individuals lacking *Cf-4* and *Cf-9* function.

Generation of molecular markers at the Milky Way locus: A cleaved amplified polymorphic sequence (CAPS) marker named MW3, located 3' of the *LoxR* gene in the MW locus, was used to distinguish the Cf0, Cf4, and Cf9 haplotypes (Figure 1). Amplification with the primer combination *LoxR-F1* and *LoxR-R1* (Table S1, supplementary information at <http://www.genetics.org/supplemental/>) yields a 496-bp product from Cf0, a 408-bp product from Cf4, and a 496-bp product from Cf9. Digestion with *HincII* generates 262- and 234-bp fragments derived from the Cf0 allele and 262 and 146 bp from the Cf4 allele, while the Cf9 allele is not cut with this enzyme. The CAPS marker MW5 was used to distinguish the Cf4 and Cf9 haplotypes at the 5' end of the MW locus (Figure 1). Amplification with the primers 99A8, 99A10R, and 99A9R generates a 601-bp product from Cf4, which is not cleaved by *RsaI*, while a 611-bp product is generated from Cf9, which is cleaved by *RsaI* to produce fragments of 502 and 109 bp. This primer combination does not amplify a product from the Cf0 haplotype. The CAPS marker MW1 was used to delimit recombination events in tract II (Figure 1), which includes the *Hcr9-4C* and *Hcr9-9E* 5' flanking regions (Figure 4B). Amplification with 99DE1F and 99DE2R (Table S1) gave products of 1.512 and 1.467 kbp from Cf4 and Cf9, respec-

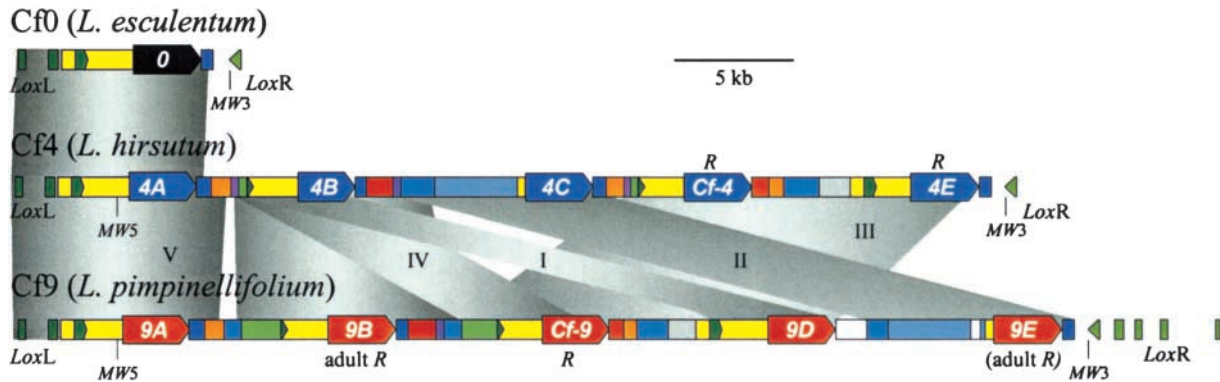


FIGURE 1.—Schematic of *Hcr9*'s in the Cf0, Cf4, and Cf9 haplotypes to summarize sequence homologies in the intergenic regions. Tracts of near-identical intergenic sequences are depicted in the same colors. The protein-coding regions of *Hcr9*'s are colored according to the haplotype from which they originated, *i.e.*, black, Cf0 from *L. esculentum*; blue, Cf4 from *L. hirsutum*; and red, Cf9 from *L. pimpinellifolium*. The regions of maximum intergenic sequence homology are indicated by the gray tracts labeled I–V. Lipoxygenase exons are indicated by green arrows and boxes. The locations of two polymorphic regions for which the CAPS markers MW3 and MW5 were developed at the 3' and 5' end of the *Milky Way* locus are indicated. Functional resistance genes with known specificities are marked with an “R,” or “adult R” in the case of *Hcr9-9B* and *Hcr9-9E*, which confer resistance in adult plants (PARNISKE *et al.* 1997; PANTER *et al.* 2002).

tively, but only the Cf9 allele can be digested by *Ava*I to generate fragments of 502 and 965 bp.

DNA gel blot analysis: Tomato genomic DNA was prepared as previously described (THOMAS *et al.* 1997) and probed with a 5' or 3' fragment of the *Cf-9* gene prepared by PCR amplification of plasmid pCDNAL9 (JONES *et al.* 1994), using the primer combinations F10/F5 or F6/Cf4-16, respectively (Figure 4B, Table S1).

DNA sequence analysis: Mutant *Cf-4* and *Cf-9* alleles were amplified from genomic DNA using gene-specific primers. The primer combination F10/F5 (Table S1) amplifies a 1.377-kbp fragment from the 5' half of *Cf-4* and a 1.568-kbp fragment from the 5' half of *Cf-9*. The primer combination F6/Cf4-16 (Table S1) amplifies a 1.500-kbp fragment from the 3' half of *Cf-4* or *Cf-9*. The amplified gene fragments were directly sequenced on both strands. The location of mutations was verified by direct sequencing of a PCR product encompassing the modified nucleotide. *Hcr9* promoter sequences were aligned and analyzed as previously described (PARNISKE *et al.* 1997; PARNISKE and JONES 1999). The *Lasergene* package (DNASTAR, Madison, WI) was used for DNA sequence assembly and primer selection.

RESULTS

Isolation and characterization of EMS-induced *Cf-9* and *Cf-4* mutants: As part of a structure-function analysis of the Cf-9 and Cf-4 proteins, and to identify extragenic suppressors of Cf gene function (DIXON *et al.* 2000), we mutagenized the Cf9 and Cf4 NILs. Approximately 2000 batches of 25 M₂ seed collected from individual Cf9 M₁ plants were screened for suppressors of PVX:*Avr9*-induced plant death (HAMMOND-KOSACK *et al.* 1995). Also, 600 M₂ families, derived from individual Cf4 M₁ plants, were screened for suppression of PVX:*Avr4*-induced plant death (THOMAS *et al.* 1997).

Four Cf9 M₂ families (S568, S315, S87, and F965) in which ~25% of the seedlings did not exhibit plant death

were identified (Table 1). Only one Cf4 M₂ family (H249) containing ~25% survivors was identified (Table 1). These mutants were investigated genetically by testcrossing to Cf0 and backcrossing to Cf9 or Cf4 (Table 1). Progeny of the four Cf9 mutants backcrossed to Cf9 and the single Cf4 mutant backcrossed to Cf4 were infected with the appropriate PVX:*Avr* construct. All of the progeny exhibited an Avr-dependent necrotic phenotype (Table 1). This analysis suggests that all five are recessive loss-of-function mutants (Table 1). None of the progeny from testcrosses between the mutants and Cf0 exhibited plant death after infection with recombinant PVX (Table 1). Therefore all five mutations map to the introgressed region within the Cf4 and Cf9 NILs that contain the corresponding Cf genes. The *Cf-4* and *Cf-9* alleles from each mutant were characterized by PCR amplification and DNA sequence analysis (Table 1).

Four recessive *Cf-9* mutants isolated in a previous study were also characterized (HAMMOND-KOSACK *et al.* 1994b). All nine mutants contained single-nucleotide substitutions within *Cf-4* or *Cf-9* or deletions encompassing *Cf-9* (Table 1). Of the nine mutations characterized five would result in single-amino-acid changes in Cf-9 (*Cf-9*^{D365N}, *Cf-9*^{D508N}, *Cf-9*^{S675L}, and *Cf-9*^{G825R}) or Cf-4 (*Cf-4*^{E662V}, see Table 1 and Figure 2). Two *Cf-9* mutant alleles could not be amplified by PCR (*Cf-9*^{Δ1} and *Cf-9*^{Δ2}) and were analyzed on DNA gel blots (results not shown). Both mutants originated from sequence deletions at the *Cf-9* locus. In the case of *Cf-9*^{Δ2} a deletion of ~300 bp that included the 3' terminal sequences of *Cf-9* occurred. In *Cf-9*^{Δ1} a more extensive deletion that included *Cf-9* and the adjacent paralog *Hcr9-9D* was observed (results not shown).

Two *Cf-9* partial loss-of-function mutants that were reported previously were also investigated (*Cf-9*^{M164} and

TABLE 1
Genetic and molecular analysis of EMS-induced mutants

M ₂ family	Cross	Ratio of necrotic:wild-type seedlings ^a	Mutated gene	Mutation	Mutant allele
S568 ^b	S568 × Cf9	24:0	<i>Cf-9</i>	Deletion	<i>Cf-9</i> ^{Δ1}
	S568 × Cf0	0:23			
S315 ^b	S315 × Cf9	20:0	<i>Cf-9</i>	G2473A	<i>Cf-9</i> ^{G825R}
	S315 × Cf0	0:21			
S87 ^b	S87 × Cf9	23:0	<i>Cf-9</i>	C2391T	<i>Cf-9</i> ^{Q734Ter}
	S87 × Cf0	0:22			
F965 ^b	F965 × Cf9	23:0	<i>Cf-9</i>	G1093A	<i>Cf-9</i> ^{D365N}
	F965 × Cf0	0:25			
H249 ^b	H249 × Cf4	24:0	<i>Cf-4</i>	T1986A	<i>Cf-4</i> ^{E662V}
	H249 × Cf0	0:23			
M140 ^c			<i>Cf-9</i>	G1521A	<i>Cf-9</i> ^{D508N}
M339 ^c			<i>Cf-9</i>	A1744T	<i>Cf-9</i> ^{R582Ter}
M2 ^c			<i>Cf-9</i>	C2026T	<i>Cf-9</i> ^{S675L}
M466 ^c			<i>Cf-9</i>	Deletion	<i>Cf-9</i> ^{Δ2}

^a Ratio of necrotic:wild-type seedlings after infection with PVX:*Avr9* or PVX:*Avr4*. Mutants were identified in the M₂ family shown.

^b This study.

^c HAMMOND-KOSACK *et al.* (1994b).

Cf-9^{M525}; HAMMOND-KOSACK *et al.* 1994b). However, no nucleotide mutations in the *Cf-9* coding sequences, or within 1.5 kbp of their 5' and 3' flanking regions, were detected.

Meiotic stability of *Cf-4*: *Cf-9* has been shown to be meiotically stable (PARNISKE *et al.* 1997). *Cf-4* stability was determined by testcrossing Cf4 (as female parent) to the Cf0 *Avr4*, *Avr9* line (Figure 3A). Eleven survivors (S₁ seedlings) were recovered from 3847 testcross progeny and allowed to self-pollinate. None of the S₂ progeny were resistant to kanamycin, suggesting they lacked both *Avr* transgenes. This was confirmed by backcrossing S₂ plants to Cf4. None of the testcross progeny exhibited the seedling lethal phenotype (results not shown). These survivors were possibly due to self contaminants or gynogenesis (*i.e.*, the development of an embryo from an egg cell without fertilization, see below).

Cf-4 stability was measured previously in an experiment to isolate *Cf-4* by transposon tagging (TAKKEN *et al.* 1998). This was analyzed in a line containing the maize transposon *Dissociation* located 3 cM proximal to *Cf-4* (TAKKEN *et al.* 1998). Three disease-sensitive mutants in 20,000 progeny were attributed to deletions or unequal crossing over at the *Cf-4* locus. These events were most likely due to the presence of the transposon. Therefore, it appears that *Cf-4* and *Cf-9* (PARNISKE *et al.* 1997) are meiotically stable in homozygous lines.

Genetic selection for novel haplotypes at the *Cf-4/Cf-9* locus: We used a facile genetic selection to identify recombinants lacking *Cf-4* and *Cf-9* on the basis of an *Avr4* and *Avr9*-dependent seedling lethal assay (HAMMOND-KOSACK *et al.* 1994a; THOMAS *et al.* 1997). *Cf-4/*

Cf-9 *trans*-heterozygotes were testcrossed to the Cf0 *Avr4*, *Avr9* line (Figure 3B). In most testcrosses *Cf-4/Cf-9* *trans*-heterozygotes were used as female parents since recombination is higher in the female gametophyte than in the male gametophyte (DE VICENTE and TANKSLEY 1991). In total, 42 S₁ survivors were recovered from 17,601 testcross progeny. These plants were characterized genetically.

The progeny of seven S₁ plants (H822, H881, H888, H889, H891, H896, and J091) from crosses where *Cf-4/Cf-9* *trans*-heterozygotes were used as female parents segregated 15:1 for kanamycin resistance, suggesting they arose from a testcross. These S₁ plants were tested for functional *Avr4* and *Avr9* transgenes by testcrossing to Cf4 and Cf9 NILs. The progeny segregated 1:1 for wild-type and seedling lethal phenotypes, confirming that each S₁ parent contained a functional *Avr4* and *Avr9* transgene (Table S3 at <http://www.genetics.org/supplemental/>).

Two additional S₁ survivors (G640 and G641) arose from crosses where *Cf-4/Cf-9* *trans*-heterozygotes were used as male parents. The S₂ progeny of these plants also segregated 15:1 for resistance to kanamycin (Table S2 at <http://www.genetics.org/supplemental/>). To confirm that these testcross survivors were not due to silencing of the *Avr4* or *Avr9* transgenes, S₂ seedlings were infected with PVX:*Avr4* and PVX:*Avr9* (Table S4 at <http://www.genetics.org/supplemental/>). No necrotic S₂ seedlings were observed, whereas Cf4 and Cf9 controls died, demonstrating that G640 and G641 must also lack *Cf-4* and *Cf-9*.

The progeny of 26 S₁ survivors were all sensitive to

Variable	A	MDCVKLVFLMLYTFLCQLALSSS			
	B	LPHLCPEDQALSLLQFKNMFTINPNASDYCYDIR TYVDIQSYPRTL SWNK STSCCSWDGVHCEDETTGQ			
			<u>xxLxLxx</u>		
	1	V	IALDLRC	SQLQGKFHSNSS	
	2	LFQLSNL	KRLDLSF	NNFTGSLISPK	
	3	FGEFSNL	THLDLSH	SSFTGLIPSE	
	4	ICHLSKL	HVLRICD	QYGLSLVPYNFELL	
	5	LKNLTQL	RELNLES	VNISSTIPS	
	6	<u>NFSSHL</u>	TTLQLSG	TELHGILPER	
	7	VFHLSNL	QSLHLSV	NPQLTVRFPTTK	
	8	<u>WNSSASL</u>	MTLYVDS	VNIADRIKPS	
	9	FSHLTSL	HELYMGR	CNLSGPIPKP	
	10	LWNL TNI	VFLHLGD	NHLEGPISH	Mutations that abolish Cf-9 function
	11	FTIFEKL	KRLSLVN	NNFDGGLEF	
	12	LSFNTQL	ERL D LSS	NSLTGPIPS	D365N
	13	<u>NISGLQNL</u>	ECLYLSS	NHLNGSIPSW	
	14	IFSLP SL	VELDLSN	NTPSGKIQE	
	15	FKS KTL	SAVTLKQ	NKLGRIKPS	
	16	LLNQKNL	<u>Q</u> ILLLSH	NNISGHISSA	L457F
	17	ICNLKTL	ILLDLGS	NNLEGTIPQCV	
	18	VERNEYL	SHL D LSK	NRLSGTINTT	D508N
	19	FSVGNIL	RVISLHG	NKLTGKVPRS	
	20	MINCKYL	ILLDLGN	NMLNDTFPNW	
21	LGYLFQL	KILSL RS	NKLGPIKSSGN	R582Ter	
22	TNLFMGL	QILDLS	NGFSGNLPERI		
23	LGNLQTM	KEIDEST			
Conserved		GFPEYISDPYDIYNYLTTISTKGDYDS			
	24	VRILDSN	MIINL SK	NRFEHGIPSI	S675L
	25	IGDLVGL	RTLNL SH	NVLEGHIPAS	
	26	FQNL SVL	<u>S</u> SLDLSS	NKISGEIP Q	E662V (in Cf-4)
	27	LASLTFL	EVLNL SH	NHLVGCIPKG	Q734Ter
	D	KQFDSFGNTSYQGNDGLRGFPLSKLCGG			
	E	EDQVTTPAELDQEEEEEDSPMISWQ			
F	GVLVGYGCGLVI S LSVIYIMWSTQYPAWFS		G825R		
G	RMDLKLEHIITTKMKKHKKRY				

FIGURE 2.—The predicted amino acid sequence of Cf-9 is shown with structural domains indicated on the left (RIVAS and THOMAS 2002). The locations of six EMS-derived mutations that abolish Cf-9 function and a single mutation that abolishes Cf-4 function are highlighted in black. The predicted amino acid changes are shown on the right of the protein sequence. The L457F change in which the F from Cf-4 replaces the L from Cf-9 abolishes Cf-9 function in *Agrobacterium*-mediated transient assays in mature tobacco leaves and is highlighted in gray (VAN DER HOORN *et al.* 2001a; WULFF *et al.* 2001). Sequences that form part of the putative β -strand/ β -turn conserved structural motif in LRR proteins (xxLxLxx, where L is leucine and x is any amino acid) are shown delimited by the dashed box. Putative N-linked glycosylation sites (NxS/T) are shown underlined.

kanamycin and further genetic tests showed they lacked functional *Avr4* and *Avr9* transgenes (results not shown). These plants arose in experiments where *Cf-4/Cf-9* trans-heterozygotes were used as female parents and were most probably due to self-pollination.

Seven other S_1 plants were completely sterile and did not produce S_2 seed. Molecular analysis and functional NPT assays suggested that one survivor may be due to androgenesis in one case (*i.e.*, the development of an embryo containing only paternal chromosomes) and gynogenesis in the others (results not shown). The rate of gynogenesis for tomato was previously estimated as $1.2\text{--}4.2 \times 10^{-4}$ (ECHOCHARD *et al.* 1969; KOORNEEF *et al.* 1989; HAMZA *et al.* 1993), which is similar to the rate observed here (2×10^{-4}). Also, the sterility observed in this class of survivors has also been attributed to gynogenesis (ECHOCHARD *et al.* 1969; IVANOVA *et al.* 2000).

In summary, of 42 S_1 plants recovered from 17,601 *Cf-4/Cf-9* testcross progeny, only 9 originated from controlled crosses (H822, H881, H888, H889, H891, H896,

J091, G640, and G641), retained functional *Avr4* and *Avr9* transgenes, and lacked *Cf-4* and *Cf-9* (Tables S2–S4).

Molecular characterization of novel haplotypes: The nine S_2 populations were analyzed with CAPS markers to determine if loss of *Cf-4* and *Cf-9* was associated with flanking marker exchange. The CAPS markers MW3 and MW5 were developed to distinguish the Cf0, Cf4, and Cf9 haplotypes at the 3' and 5' ends of the MW locus, respectively (Figure 1 and MATERIALS AND METHODS). All survivors contained sequences from the Cf4 haplotype at the 5' end of the MW locus and sequences from the Cf9 haplotype at the 3' end of the MW locus (Table 2). The simplest explanation for these data is that loss of *Cf-4* and *Cf-9* resulted from crossing over at the *Cf-4/Cf-9* locus. The analysis enabled identification of individuals homozygous for the recombinant chromosome that could be used to determine their *Hcr9* composition.

DNA gel blots, PCR amplification, and DNA sequencing were used to locate the crossovers in the homozy-

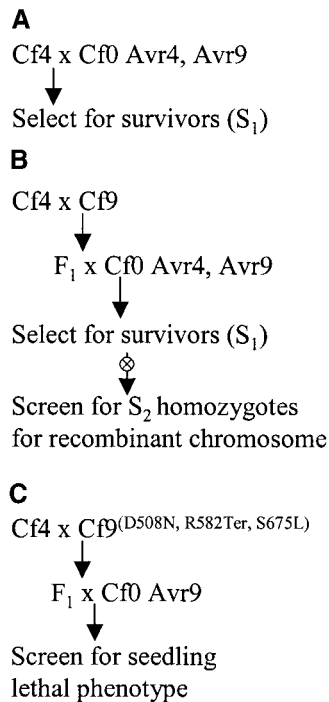


FIGURE 3.—Crossing strategies used in this study. (A) The meiotic stability of *Cf4* was measured by testcrossing to the Cf0 Avr4, Avr9 line. Most of the progeny display the seedling lethal phenotype as a result of Avr4-induced seedling death. Survivors (S) can be recovered when *Cf4* is mutated or lost due to unequal crossing over or gene conversion. (B) Crossing strategy to identify loss-of-function alleles or recombinants lacking *Cf4* or *Cf9* function in a *Cf4/Cf9* *trans*-heterozygote. (C) Crossing strategy to recover functional alleles of EMS-derived *Cf9* mutants.

gotes. Genomic DNA was digested with *Bgl*II, blotted, and hybridized with a probe from the 5' half of *Cf9*. All nine recombinants lacked *Bgl*II fragments from *Cf4* and *Cf9* (Figure 4A) and a 6.6-kbp *Bgl*II fragment derived from *Hcr9-4C*. All retained a 4.1-kbp *Bgl*II fragment derived from *Hcr9-4B* and *Bgl*II fragments derived from *Hcr9-9E* (Figure 4, A and B). Therefore, the crossovers in the new recombinants appear to be located within the *Hcr9-4B* to *Hcr9-4C* region within the Cf4 haplotype and in the *Hcr9-9D* to *Hcr9-9E* region within the Cf9 haplotype. This is a region of highly homologous DNA sequence that contains the 5' flanking region and coding sequences of *Hcr9-4C* and *Hcr9-9E* (Figures 1 and 4B).

Two distinct classes of recombinants were discerned on the basis of their characteristic *Hcr9-9E*-derived *Bgl*II fragments. Five recombinants (H882, H888, H891, H896, and J091) retained a 3.6-kbp *Bgl*II *Hcr9-9E* fragment present in the Cf9 haplotype (Figure 4A). The remaining recombinants (G640, G641, H881, and H889) contained a novel *Hcr9-9E* *Bgl*II fragment of ~3.2 kbp (Figure 4A). This difference is due to a 353-bp deletion in the *Hcr9-4C* 5' flanking DNA compared to the corresponding *Hcr9-9E* flanking region (Figure 4B). Cross-

overs located downstream of this deletion contain a chimeric *Bgl*II fragment of ~3.2 kbp containing the *Hcr9-9E* coding region (Figure 4B). The crossovers in these latter recombinants could thus be delimited to a 2.289-kbp region downstream of the deletion in the *Hcr9-4C* 5' flanking DNA and upstream of the *Hcr9-9E* coding sequence (Figure 4B).

Crossovers in the other recombinants (H882, H888, H891, H896, and J091) were delimited to a region 3' to the *Hcr9-4B* gene in the Cf4 haplotype. The CAPS marker MW1 (Figure 4B) was used to further delimit the crossovers in these recombinants. All five recombinants retained MW sequences derived from the Cf4 haplotype (Table 2 and Figure 4). This delimited the crossovers to a 3.6-kbp region between MW1 and the 353-bp insertion in the *Hcr9-9E* 5' flanking region within the Cf9 haplotype (Figure 4B). DNA fragments spanning these regions were PCR amplified and sequenced. The crossovers were then localized to a region between the two closest flanking polymorphic nucleotides (Table 2). Some crossovers in class II recombinants are coincident with AT (V408, H881) and ATT (V517, V512, H891, and J091) microsatellite repeats (Figure 4C).

Of the nine recombinants reported here five were similar to the previously reported class IIa intergenic recombinants (H822, H888, H891, H896, and J091) and four were similar to the previously reported class IIb intergenic recombinants (G640, G641, H881, and H889—see Table 2; PARNISKE *et al.* 1997; THOMAS *et al.* 1997). The crossovers in G640 and G641 were each delimited to a 192-bp region that extended 178 bp into the 5' region of the *Hcr9-4C* and *Hcr9-9E* open reading frames. However, this did not result in any nucleotide substitutions in the *Hcr9-9E* gene. When the reciprocal products of the class II recombinants are considered (*i.e.*, the haplotypes that carry both *Cf4* and *Cf9* and five additional *Hcr9*'s) and the number of progeny screened, the recombination frequency in the 3.6-kbp region delimited by these recombinants is 35 kbp/cM. This number is ~20 times higher than the genome average for tomato of 740 kbp/cM (TANKSLEY *et al.* 1992) and therefore represents a recombination "hot-spot" in this cross.

In this genetic selection, the loss of *Cf4* or *Cf9* gene function as a result of unequal crossing over was 1 in 1955, which is not significantly different from the rate of 1 in 1500 ($\chi^2 = 0.64$) obtained in the screen for disease sensitivity using *C. fulvum* race 5 (PARNISKE *et al.* 1997; THOMAS *et al.* 1997).

Measuring *Cf9* intragenic recombination in *Cf4/Cf9 trans*-heterozygotes: No *Hcr9* intragenic recombination events have been observed in the progeny of *Cf4/Cf9 trans*-heterozygotes. We used a genetic screen to identify intragenic recombinants in *trans*-heterozygous plants containing *Cf4* and EMS-induced mutant alleles of *Cf9*. The *trans*-heterozygous plants were testcrossed to a Cf0 Avr9 line (Figure 3C). Intragenic recombinants between

TABLE 2
Selection and molecular analysis of homozygous S₂ recombinants

S ₁ (survivor)	Genotype at MW ^a		Genotype at MW1	Recombinant class ^b	Recombination interval (bp)	Location 5' of ATG of <i>Hcr9-9E</i>
	5'	3'				
G640	<i>Cf-4</i>	<i>Cf-9</i>	<i>Cf-4</i>	IIb	190	13
G641	<i>Cf-4</i>	<i>Cf-9</i>	<i>Cf-4</i>	IIb	190	13
H822	<i>Cf-4</i>	<i>Cf-9</i>	<i>Cf-4</i>	IIa	14	3546
H881	<i>Cf-4</i>	<i>Cf-9</i>	<i>Cf-4</i>	IIb	75	494
H888	<i>Cf-4</i>	<i>Cf-9</i>	<i>Cf-4</i>	IIa	144	2015
H889	<i>Cf-4</i>	<i>Cf-9</i>	<i>Cf-4</i>	IIb	116	361
H891	<i>Cf-4</i>	<i>Cf-9</i>	<i>Cf-4</i>	IIa	49	2954
H896	<i>Cf-4</i>	<i>Cf-9</i>	<i>Cf-4</i>	IIa	211	3762
J091	<i>Cf-4</i>	<i>Cf-9</i>	<i>Cf-4</i>	IIa	165	3311
V514 ^d	<i>Cf-4</i>	<i>Cf-9</i>	<i>Cf-9</i>	I	124	1266 ^c
V517 ^d	<i>Cf-4</i>	<i>Cf-9</i>	<i>Cf-4</i>	IIa	50	2904
V512 ^d	<i>Cf-4</i>	<i>Cf-9</i>	<i>Cf-4</i>	IIa	49	2954
V516 ^d	<i>Cf-4</i>	<i>Cf-9</i>	<i>Cf-4</i>	IIa	21	3394
V408 ^d	<i>Cf-4</i>	<i>Cf-9</i>	<i>Cf-4</i>	IIb	75	494

^a Based on CAPS analysis with MW5 and MW3; see Figure 1.

^b Based on DNA gel blot analysis; see Figure 4A.

^c 5' of ATG of *9D*.

^d Reported in PARNISKE *et al.* (1997) and THOMAS *et al.* (1997).

Cf-9 and another *Hcr9* (such as *Cf-4*) could reconstitute a functional *Cf-9* gene that would be identified as a necrotic individual. *Cf-4* and *Cf-9* contain several kilobase pairs of homologous 5' and 3' flanking DNA (tract III in Figure 1). As *Cf-4* and *Cf-9* encode the same amino acid sequence in the last 1 kbp of their 3' halves, *in vivo* intragenic recombinants in this region would be expected to be functional. Before commencing the screen, it was established that sufficient DNA could be isolated for functional characterization of the chimeric gene from seedlings showing early signs of necrosis.

To identify intragenic recombinants *Cf4* was crossed to three different *Cf-9* mutants (*Cf-9*^{D508N}, *Cf-9*^{R582Ter}, and *Cf-9*^{S567L}—see Table 1 and Figure 2). All of the mutations in *Cf-9* are located downstream of the amino acid L457. This residue is the most C-terminal variant amino acid that distinguishes *Cf-9* from *Cf-4* that is required for Avr9 recognition (VAN DER HOORN *et al.* 2001a; WULFF *et al.* 2001). The *trans*-heterozygotes were testcrossed to *Cf0* Avr9 plants and the progeny were inspected for gain-of-function alleles (Table 3). No functional *Cf-9* alleles were recovered in the 15,652 progeny tested (Table 3).

DISCUSSION

Characterization of EMS-induced mutants of *Cf-9* and *Cf-4*: Six EMS-induced point mutants of *Cf-9* and one *Cf-4* mutant were characterized at the molecular level. Two mutants affecting LRRs 21 and 26 (*Cf-9*^{R582Ter} and *Cf-9*^{Q734Ter}) would result in the production of truncated *Cf-9* proteins lacking the C-terminal domains D, E, F,

and G (Figure 2). These domains are essential for *Cf-9* function (THOMAS *et al.* 2000). The *Cf-4*^{E662V} mutant would result in the substitution of a putative solvent-exposed amino acid in the *xxLxLxx* motif within LRR 26 (Figure 2).

The *Cf-9*^{G825R} mutant would result in substitution of an aliphatic amino acid with a positively charged amino acid in the hydrophobic α -helical transmembrane domain. The G825 residue forms part of a tri-tandem GXXXG motif (see Figure 2) that is required for homo- and heterodimerization of membrane proteins *in vivo* (GERBER and SHAI 2001). Therefore, the mutant protein may not localize in the membrane or may be impaired in its interaction with another protein required for *Cf-9*-dependent signaling.

The other mutations (*Cf-9*^{D365N}, *Cf-9*^{D508N}, and *Cf-9*^{S675L}) would result in substitution of single putative solvent-exposed amino acids within the *xxLxLxx* motif in *Cf-9* C-terminal LRRs (Table 1 and Figure 2). These mutations could also alter the glycosylation pattern within *Cf-9* (Figure 2). The protein encoded by the *Cf-9*^{D365N} mutant allele would contain a substituted amino acid within the two additional N-terminal LRRs of *Cf-9* (compared to *Cf-4*) that are required to induce an Avr9-dependent hypersensitive response (VAN DER HOORN *et al.* 2001a,b; WULFF *et al.* 2001). With the exception of the L457 residue in *Cf-9* LRR 16, this is the only other example of a single-amino-acid substitution in the N-terminal LRRs that completely abolishes *Cf-9* function (VAN DER HOORN *et al.* 2001a; WULFF *et al.* 2001). However, it is not yet known if any of these variant alleles encode stable proteins *in planta*. The *Cf-9*^{D508N}, *Cf-9*^{S675L},

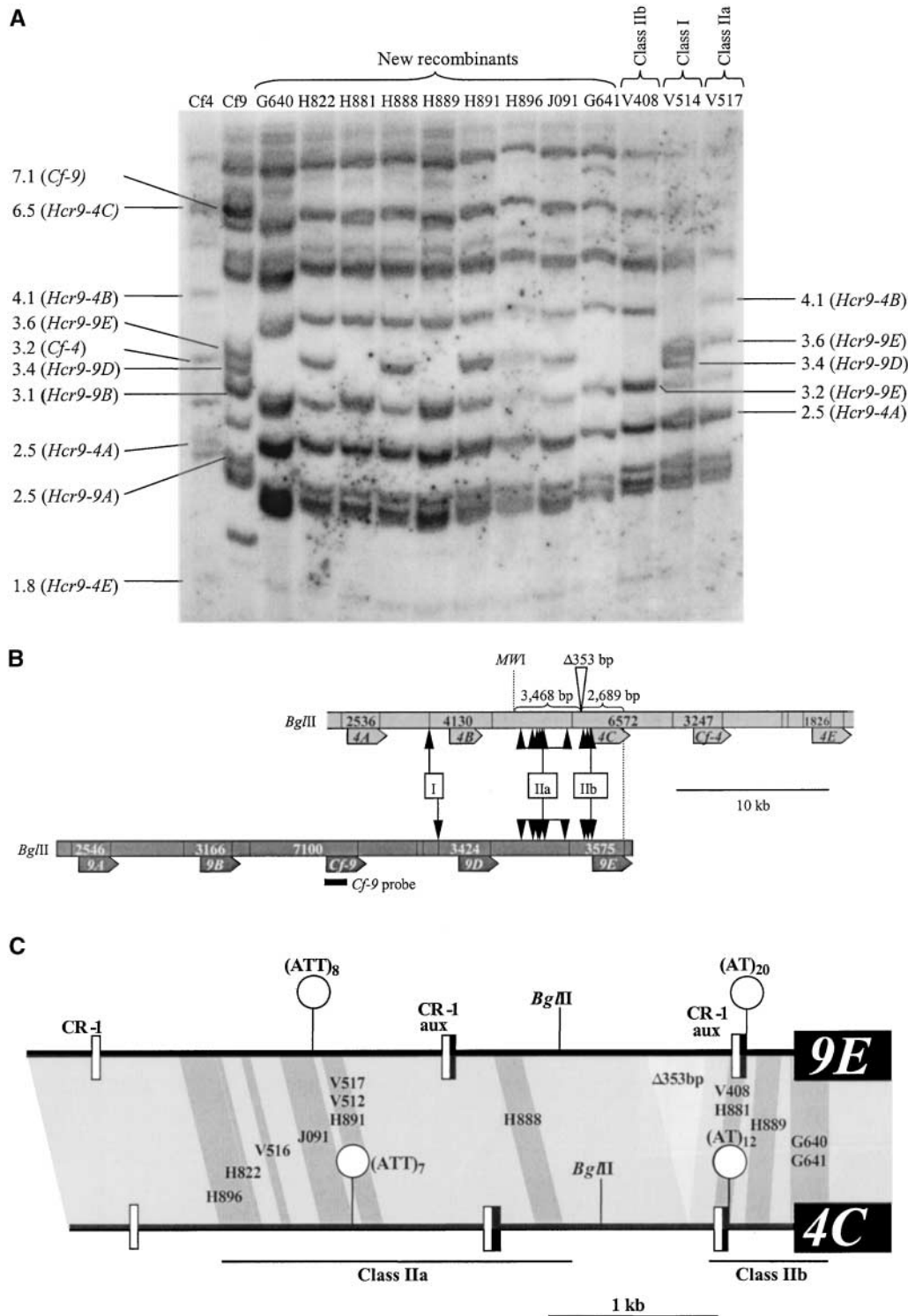


FIGURE 4.—(A) Analysis of Cf4 and Cf9 NILs and S_2 survivors. DNA gel blots of F_2 plants homozygous for the recombinant chromosome, plus DNA from three plants that define three recombinant classes, class I (V514), class IIa (V517), and class IIb (V408; PARNISKE *et al.* 1997; THOMAS *et al.* 1997), are shown. DNA was digested with *Bgl*II and hybridized with a 1.6-kbp probe from the 5' end of *Cf-9*. The sizes of *Bgl*II DNA fragments in kilobase pairs that originate from specific *Hcr9*'s are indicated on the left and the right. (B) Physical organization of the Cf4 (light shading) and Cf9 (dark shading) haplotypes. *Bgl*II restriction sites are represented by vertical lines and the sizes of fragments revealed upon hybridization to the *Cf-9* probe are indicated in base pairs. *Hcr9* protein-coding regions are indicated by horizontal arrows. The crossovers in previously characterized recombinants (V408, V512, V514, V516, and V517; PARNISKE *et al.* 1997) and recombinants analyzed in this study are indicated by solid triangles. On the basis of the comparison between the restriction patterns of the previously characterized *Cf-4/Cf-9* disease-sensitive recombinants (THOMAS *et al.* 1997) and the recombinants reported here, the crossovers in S_1 survivors were localized to a 3.468-kbp region delimited by the CAPS marker MW1 and a deletion of 353 bp in *Hcr9-4C* (class IIa) or a contiguous fragment of 2.289 kbp (class IIb). (C) Expanded view of crossovers in the *Hcr9-4C/Hcr9-9E* 5' flanking regions. Crossovers are indicated by darkly shaded bands. The lightly shaded background represents regions with >98.5% sequence identity. The 5' ends of the coding regions of *Hcr9-9E* (9E) and *Hcr9-4C* (4C) are indicated. AT n and ATT n microsatellite repeats are indicated by lollipops and fragments of DNA with homology to a dispersed repeat element (CR-1, 92% identity in 38 nucleotides) associated with the *Cab-1* locus of tomato and auxin-responsive promoter element (aux, 90% identity in 40 nucleotides) from tobacco (BERNATZKY *et al.* 1988; TAKAHASHI *et al.* 1990) are indicated by open and solid boxes, respectively.

TABLE 3
Screening for restoration of Cf-9 function

	Progeny screened	Necrotic seedlings ^a	Functional recombination interval ^b (bp)
(<i>Cf-9</i> ^{D508N} / <i>Cf-4</i>) × Avr9	5,450	0	150
(<i>Cf-9</i> ^{R582Ter} / <i>Cf-4</i>) × Avr9	3,894	0	372
(<i>Cf-9</i> ^{S675L} / <i>Cf-4</i>) × Avr9	6,308	0	654
Total:	15,652	0	

^a Necrotic seedlings arising due to intragenic recombination.

^b Assuming recombination between *Cf-9* and *Cf-4* through tract III; Figure 1. The distance in base pairs between the point mutation in the corresponding mutant allele of *Cf-9* and the sequence encoding the L457F Cf-9 specificity determinant is shown.

Cf-9^{G825R}, and *Cf-4*^{E662V} mutations affect amino acids that are conserved in the C-terminal LRRs of all predicted Hcr9 and Hcr2 proteins analyzed (DIXON *et al.* 1996, 1998; PARNISKE *et al.* 1997; PARNISKE and JONES 1999). These residues may be important for interacting with signaling partner proteins (DIXON *et al.* 1996), or they may affect protein stability.

No extragenic suppressors of *Cf-9* or *Cf-4* function were identified, in contrast to a previous screen (HAMMOND-KOSACK *et al.* 1994b) and mutagenesis of the Cf2 line, which identified *Rcr3* (DIXON *et al.* 2000). The high doses of Avr4 and Avr9 elicitor delivered by recombinant PVX may preclude the identification of mutations that partially compromise *Cf-9* or *Cf-4* function. The identification of extragenic suppressors of *Cf-4* and *Cf-9* may require more sensitive screens that could reveal partial loss-of-function alleles.

A recombination hotspot in *Cf-4/Cf-9* trans-heterozygotes: This selection identified recombinant *Hcr9* haplotypes similar to our previous genetic screen (PARNISKE *et al.* 1997). Molecular analysis revealed flanking marker exchange in the progeny lacking *Cf-4* and *Cf-9*. Conceivably, this was due to gene conversion and the conversion tracts extended >12.5–15.9 kbp beyond the 5' marker or 4.2–8.0 kbp beyond the 3' marker (Figure 1). However, this is unlikely since the ratio of gene conversions to crossovers is low in plants (DOONER and MARTÍNEZ-FÉREZ 1997; SCHNABLE *et al.* 1998; CHIN *et al.* 2001). In plants, homologous but unequal crossing over does not appear to be associated with gene conversion, and this is analogous to the situation in humans (METZENBERG *et al.* 1991).

Thirteen of the 14 crossovers were localized within tract II, in the 5' flanking regions of *Hcr9-4C* and *Hcr9-9E*, while only one was located in tract I (Figures 1 and 4B). The 13 crossovers in tract II are clustered in two groups within a 3.6-kbp region (Figure 4, B and C). The apparent preference for tract II over tract I may be due to the fact that it is significantly longer. This would be

consistent with results from other studies in humans and plants (METZENBERG *et al.* 1991; DOONER and MARTÍNEZ-FÉREZ 1997).

Recombination hotspots in plants usually occur within genes (BROWN and SUNDARESAN 1991; EGGLESTON *et al.* 1995; BÜSCHGES *et al.* 1997; DOONER and MARTÍNEZ-FÉREZ 1997; OKAGAKI and CLIFFORD 1997), but an intergenic recombination hotspot has been reported in the multigenic *a1-sh2* interval of maize (YAO *et al.* 2002). The recombinants in this study define a hotspot in an *Hcr9* intergenic region where the recombination frequency is 20 times the genome average (35 kbp/cM). In tomato, recombination frequencies on the short arm of chromosome 1 can vary by one order of magnitude in different interspecific crosses (BONNEMA *et al.* 1997).

The most extensive regions of sequence homology at *MW* are in tracts II, III, and V (Figure 1). However, a large number of the predicted recombinant classes would not be identified using the current genetic selection; *e.g.*, crossing over within tract V would not result in recombinant chromosomes lacking *Cf-4* and *Cf-9*. The most extensive tract of sequence homology is tract III that includes the 5' and 3' flanking sequences of *Cf-4* and *Cf-9* (Figure 1). Only intragenic crossovers that involved the 5' coding sequences of *Cf-4* and *Cf-9* might generate a chimeric *Hcr9* that lacked Avr4 or Avr9 recognition specificity (VAN DER HOORN *et al.* 2001a,b; WULFF *et al.* 2001). In conclusion, only a fraction of all predicted crossovers could have been identified in our selection.

Intragenic recombination at the *Cf-4/Cf-9* locus: We attempted to measure the frequency of intragenic recombination in three crosses containing *Cf-9* mutant alleles and *Hcr9-4s* (Figures 2 and 3C). No gain-of-function *Cf-9* alleles were detected in 15,600 testcross progeny (Table 3). Functional analysis of Cf-9 has shown that L457 in LRR 16 is essential for Avr9 recognition (Figure 2; VAN DER HOORN *et al.* 2001a; WULFF *et al.* 2001). In our model the most likely *Cf-9* partner for intragenic recombination would be *Cf-4* (through pair-

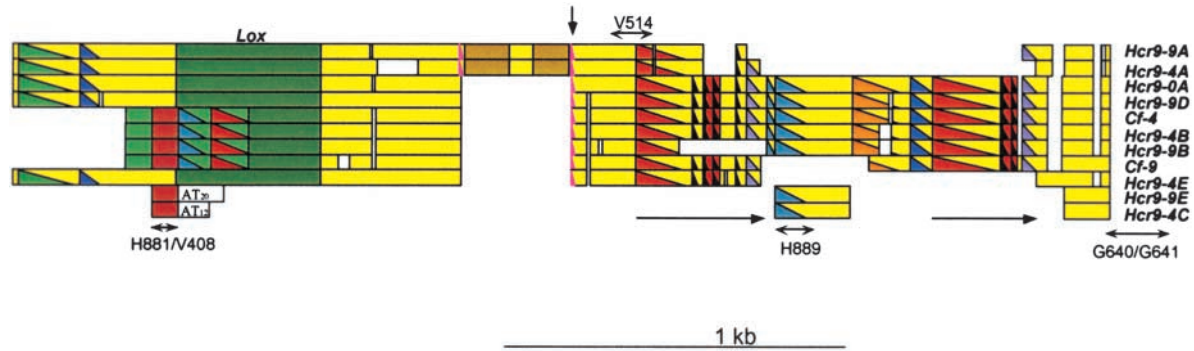


FIGURE 5.—Sequence relationships of *Hcr9* intergenic regions at the *MW* locus. Blocks of color indicate stretches of near-identical DNA sequence. Most *Hcr9* 5' flanking sequences, except *Hcr9-4C* and *Hcr9-9E*, are highly homologous, suggesting a common evolutionary origin. *Hcr9-4C* and *Hcr9-9E* share only a short region of 5' flanking sequence homology with the other *Hcr9*'s. The extant versions have most likely evolved by deletions, duplications, and insertions; *e.g.*, a deletion associated with a small direct repeat (pink triangles) is indicated by a vertical arrow. No sequence inversions are apparent as indicated by the polarity of the triangles. The two horizontal right-hand arrows represent a duplicated sequence. The locations of crossovers in *Cf-4/Cf-9* *trans*-heterozygotes are delimited by horizontal double-headed arrows relative to the ATG of *Hcr9-9E* (plants G640, G641, H889, H881, and V408) and *Hcr9-9D* (plant V514).

ing in tract III, see Figure 1), but crossovers involving other *Hcr9*'s could also create a functional allele. The regions of sequence between nucleotides encoding L457 and the lesions in the three *Cf-9* mutant alleles used in our analysis are 150, 372, or 654 bp (Table 3). Considering the interval within which crossing over would have had to occur to reconstitute a functional *Cf-9* allele, the number of progeny analyzed was too low. Even if the recombination frequency in tract III were comparable to that in tract II only one gain-of-function allele would be expected in 15,600 progeny. Therefore no clear evidence for or against intragenic crossing over in *Hcr9*'s has been provided by this study.

Comparative sequencing of the *Cf-4/Cf-9* locus suggested that gene conversion and intragenic recombination have played significant roles in generating sequence variation (PARNISKE *et al.* 1997). The crossovers in two *Cf-4/Cf-9* recombinants described here (G640 and G641—see Figure 4) included the 5' coding sequences of *Hcr9-9E* and *Hcr9-4C*, but these did not result in genes encoding novel *Hcr9*'s. However, natural populations of tomato, and particularly *L. pimpinellifolium*, appear to be extremely polymorphic at *Cf* gene loci on the short arm of chromosome 1 (LAUGÉ *et al.* 1998b, 2000). Also, the role of intragenic recombination in *Hcr9* evolution was demonstrated by analysis of natural variants of *Cf-9*. One allele that most likely arose by intragenic recombination between *Cf-9* and *Hcr9-9D* was characterized (VAN DER HOORN *et al.* 2001b).

It has been proposed that sequence differences in the *Hcr9* intergenic regions suppress mispairing between *Hcr9* paralogs, thereby preventing sequence homogenization of the gene cluster, while facilitating intragenic exchange between orthologs (PARNISKE *et al.* 1997). This would account for the apparent meiotic

stability of *Cf-9* (PARNISKE *et al.* 1997) and *Cf-4* (this study). Our detailed analysis of the 5' flanking regions of *Hcr9*'s at the *MW* locus shows that sequence duplications and insertions have been important in the evolution of the *Hcr9* 5' flanking DNAs, as indicated by the juxtaposition of numerous blocks of near-identical DNA sequences (Figure 5).

In the case of *Cf-4/Cf-9* *trans*-heterozygotes, where the genes originated from different *Lycopersicon* species, crossing over may be restricted to the regions of maximum sequence homology. High levels of recombination are observed in this cross, but it is not clear why this is apparently restricted to the intergenic regions. It is possible that the frequency of *Hcr9* intragenic recombination in this cross is below the level that could be detected by our analysis. One way to analyze the rate of intragenic recombination at this locus would be to determine the gain-of-function frequency in *Cf-9* *trans*-heterozygotes containing distinct mutant alleles (*e.g.*, excision alleles of *Dissociation*-tagged *Cf-9* mutants; JONES *et al.* 1994).

Clearly, other strategies will need to be devised to determine the molecular mechanisms of *Hcr9* evolution at this locus. Identifying the full spectrum of recombinant chromosomes will require high-throughput genetic or molecular screens that can reveal an exchange of flanking markers at the *Cf-4/Cf-9* locus.

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